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Studies on the Persistence of Corynebacterium insidiosum in Soil and of an Antagonistic Variant of the Species

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STUDIES ON THE PERSISTENCE OF CORYNEBACTERIUM INSIDIOSUM IN SOIL AND OF AN ANTAGONISTIC VARIANT OF THE SPECIES

BY

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STUDIES ON THE PERSISTENCE OF CORYNEBACTERIUM INSIDIOSUM IN SOIL AND OF AN ANTAGONISTIC VARIANT OF THE SPECIES

Abstract

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Under the Supervision of Professor George Semeniuk

Studies were conducted on the persistence in soil of Corynebacterium insidiosum, the alfalfa wilt bacterium, and on an antagonistic variant of that species. The persistence was followed by plate counting colonies from soils heavily inoculated with cells of this bacterium. The antagonistic variant of the species was studied directly and from cells or culture filtrates spot-planted on agar media containing seedings of other bacteria.

Most cells of the wilt bacterium remained viable on glass surfaces for over 7 months at different temperatures, and in moist sterilized soil for over 72 days at room temperature.

The cells persisted for only a few days in moist non-sterilized soil at room temperature, for 66 days in moist sterilized and non-sterilized soil at freezing or slightly-above freezing temperatures, and for over 5.5 months in very dry or moist soil at below-freezing temperatures.

The bacterium persisted for a longer period in some soils than in others, regardless of the texture or pH of those soils. It persisted longer in moist sterilized soil than in a similar soil contaminated with a small amount of non-sterilized soil or a streptomycete. Soil microorganisms thus were considered responsible for the rapid decline of

C. insidiosum in soil, with streptomycetes probably contributing to the decline.

The wilt bacterium did not persist over 30 days in wilt-infected alfalfa roots stored in a moist non-sterilized soil at room temperature.

The antagonistic variant of C. insidiosum was proven such from morphological, cultural, serological, pathogenic and antibiotic sensitivity tests. It inhibited other cultures of C. insidiosum, but not itself. The other cultures of C. insidiosum were non-inhibitory to the antagonistic variant or to itself; hence, such cultures were termed non-antagonists.

The inhibitory property of the antagonistic variant was exhibited by all cells of a culture, and it was stable on passage of the bacterium through alfalfa roots alone or in combination with a non-antagonistic culture of C. insidiosum. The antagonistic and non-antagonistic cells were mutually compatible in such roots.

The inhibitory intensity of the antagonistic variant varied among cells re-isolated from alfalfa roots where non-antagonistic cells were present. The intensity was less from old than from young cultures, less on peptone-containing than on peptone-deficient Burkholder's agar medium, less on heavily seeded than on lightly seeded Burkholder's agar medium, less on normal than on reduced agar concentrations in Burkholder's medium, and less at well-below optimum than at optimum incubation temperatures.

The inhibitory principle was extracellular, water-soluble and diffusible through agar. It was contained in a filtrate from Burkholder's

agar medium but not from Burkholder's broth medium. It passed through a fritted-glass but not a Seitz filter. The principle was precipitated by acetone, resistant to chloroform, not sedimented by high-speed centrifugation, and thermostabile over a pH range of 3.0 to 8.2. Short steamings of the filtrate doubled or tripled the effectiveness of the inhibitory filtrate, and rendered it susceptible to inactivation by aeration.

The inhibitory range of the antagonistic variant was limited to cultures of C. insidiosum, C. michiganense and C. sepedonicum. One non-antagonistic culture of C. insidiosum slightly inhibited cultures of C. michiganense. Four cultures of C. michiganense markedly or slightly inhibited the antagonistic variant and non-antagonistic cultures of C. insidiosum. The inhibitory effectiveness of a filtrate of one culture of C. michiganense was more than doubled by a short period of steaming.

The inhibitor of the antagonistic strain of C. insidiosum was tentatively classed with the bacteriocins because of its similarity to this group of compounds.

STUDIES ON THE PERSISTENCE OF CORYNEBACTERIUM INSIDIOSUM IN SOIL AND OF AN ANTAGONISTIC VARIANT OF THE SPECIES

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Doctor of Philosophy, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Head of the Major Department

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G.A.K.

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INTRODUCTION

Bacterial wilt of alfalfa caused by Corynebacterium insidiosum (McCulloch) Jensen is probably the most important disease of this crop in North America. It is a slow-developing vascular disease that operates over one to several years on individual plants, reducing forage yields through stunted top growth until the death of the plants. Its intensity varies from plant to plant, from one part of the field to another, from one field to another and from one part of the country to another; furthermore, the intensity is dependent upon conditions that are not well understood. Such conditions include differences in virulence of the pathogen, differences in susceptibility of the host, time and avenues of entry of the pathogen into the host, and the persistence of the pathogen in soil and other substrata. Appraisal of these conditions and the influence they have on C. insidiosum is necessary for progress in the control of the disease.

REVIEW OF LITERATURE

The economic importance of bacterial wilt of alfalfa is indicated from various reports in the United States which have shown that reductions in forage yield occur as a result of this disease. Wiant and Starr (59) found alfalfa stand negatively correlated with wilt incidence.

Although the origin of bacterial wilt of alfalfa is obscure, it is not a new disease. Prior to the first description of this disease by Jones (30), various investigators had observed the disease and made brief reports on it (10, 22, 41, 55). Shortly after the first description of the disease, Jones and Weimer (37) discovered that it was well established in the older alfalfa-growing regions of the United States. By 1926 Jones and McCulloch (35) described the disease and the causal organism more fully. The first time the disease was reported outside of the United States was in 1929 when Jones (33) isolated the pathogen from discolored alfalfa roots sent to him from Turkestan. Since 1936 bacterial wilt has been found in various parts of Western Canada. More recently the disease was reported in Chile by Gottlieb et al. (16), and in Italy by Ribaldi et al. (53).

Jones (31) observed wilt bacteria between cells of infected alfalfa plants obtained from a field at Monroe, Wisconsin in 1927. When these plants were injured by frost, the epidermis and cortex were broken and bacteria were observed to have passed through these rifts and to have adhered to the exterior. In this manner the bacteria could have been carried into the soil and washed away with the surface water. Although the means by which this pathogen makes its exit from

diseased tissue has not been fully clarified, one can be reasonably sure that any mechanical injury such as frost damage or wounding by mowers may release wilt bacteria from infected alfalfa plants into the soil.

Other studies have indicated that once in the soil, wilt bacteria are carried by surface water to healthy alfalfa plants in low-lying areas. Peltier and Jensen (48) in Nebraska, and Jones and McCulloch (35) in Wisconsin observed more diseased plants in low places in a field along natural drainways than at high places; furthermore, the finding of Jones and McCulloch (35) that the disease was more prevalent in areas with a rainfall of 25 inches or more per year than in areas with less rainfall, gives support to this means of dissemination.

Once virulent cells of C. insidiosum come in contact with susceptible plants, any mechanical injury to the roots, crowns or stems provides portals of entry for the pathogen. Peltier (46) found the alfalfa wilt bacterium incapable of entering mature alfalfa plants through any natural portals, and for this reason freshly-made wounds were necessary for entrance. Jones (31) was the first to find evidence that winter injury of alfalfa plants provided a point of entry for the pathogen. In Wyoming Wiant and Starr (59) ascertained that a decided correlation existed between winter injury and the amount of bacterial wilt. Peltier and Jensen (48) determined that infection took place more readily during May and June than at other times of the year. This period of time coincided with the first cutting of the crop and thereby indicates the importance of mower-dissemination of the causal organism.

Jones (31) found the pathogenic bacteria able to spread through the larger intercellular spaces in all directions once they had entered the plant. Along the phloem rays to the interfascicular cambium where large cells were found to be abundant, the bacterial progress was particularly rapid. As soon as the bacteria had passed the cambium, they spread along the middle lamella between the parenchymatous cells of the vascular bundles until they reached the vessels. Peltier and Schroeder (49) found the bacteria to enter the vessels through the middle lamella at the pits where the bacteria multiplied rapidly. After the bacteria entered the vessels of several bundles in the roots they moved on to the crowns and stems. Both Jones (31) and Peltier and Schroeder (49) found the wilt bacteria less abundant in the above-ground parts of the plants than in the roots. In the stem, wilt bacteria were found in the pith, some of the innermost vessels, only occasionally in the outermost xylem elements and never in the phloem. These investigators did not demonstrate the presence of wilt bacteria in alfalfa seed, and not until recent times was this accomplished by Cormack and Moffatt (4).

In early experiments by Jones (32), Peltier (45), Weimer and Madson (58) and Graber and Jones (17) the alfalfa varieties Ladak and Hardistan and introductions from Turkestan proved to be resistant to bacterial wilt. According to Haskel and Wood (21), Turkestan, while resistant, lacked good fodder quality, a characteristic which the wilt-susceptible variety Grimm possessed. Peltier and Tysdal (50) found the French variety Provence to be a new source of wilt resistance. In

Kansas Salmon (56) determined in a three year test that Provence had higher resistance than Turkestan. Ranger alfalfa which is resistant to wilt was developed in Nebraska from selections within the Turkestan, Cossack and Ladak varieties. Buffalo alfalfa, similarly derived and adapted to conditions in Kansas, is also highly resistant.

The work of Peltier and Schroeder (49) indicated that wilt resistant plants possessed narrow vessel openings and thick cell walls. Jones (34) was not in agreement with Peltier and Schroeder's findings in that he could not find anatomical differences between resistant and susceptible varieties. In resistant varieties, however, Jones found invasion of parenchymatous tissue at the point of inoculation was retarded, while in susceptible varieties invasion was very rapid.

Peltier and Tysdal (51) ascertained that wilt resistance was maintained or increased by self-fertilization of alfalfa lines up to the fifth generation, under conditions of continuous selection for wilt resistance. They found more than one genetic factor involved in resistance. Brink et al (1) selfed crosses between resistant and susceptible Hardistan plants and observed segregation in the F_2. Because they were unable to make any factorial interpretation of their data they concluded that the genetic basis for resistance to bacterial wilt was complex. Jones (34) obtained an increase in resistance in open pollinated progeny of selected plants. Crosses between the susceptible Grimm variety and highly resistant plants made by Jones and Smith (36) resulted in F_1 plants that were immune. They concluded that wilt resistance could be introduced into commercially-grown susceptible

varieties of alfalfa by hybridization techniques. Wilson (60) found resistance to wilt resolvable into terms of separate genes. Three to four partially dominant genes were isolated, differing in strength of resistance. The P gene in the homozygous state conferred 72 per cent resistance to plants. By use of the backcross method, Donnelly (9) increased the resistance of clones by incorporating into them the P gene described by Wilson. Resistance of the backcrosses, however, depended upon the genotype of the clone used as the recurrent parent.

McCulloch (40) and Jones and McCulloch (35) reported that C. insidiosum had considerable vitality and survived long exposures to sunlight, freezing and desiccation. Cells of this bacterium remained viable for 23 days when dried on cover slips. They remained alive for 5 months in infected alfalfa hay according to Jones and McCulloch (35), and Cormack et al (5) determined that they could remain viable for 3 years in infected alfalfa plants dried and maintained at -20°C, 5°C or room temperature.

If the alfalfa wilt bacterium persisted in soil for a long time, its presence there could present a peril to new seedings of alfalfa or to older disease-free stands. Jones and McCulloch (35) were unable to determine how long C. insidiosum persisted in soil; however, they did observe disease in new plantings on old fields, which seemed to spread from old diseased crowns that had survived an intervening period of cultivation. Peltier and Jensen (48) were also unable to determine the longevity of this pathogen in soil, and isolations made by them from dead plants left in the field over winter failed to yield cultures

of C. insidiosum. Jensen (28) identified an isolate of C. insidiosum from a grass soil in Australia; nevertheless, this isolate has not been proven virulent.

McCulloch (40) and Jones and McCulloch (35) first described the morphology and cultural characteristics of C. insidiosum. This species was a gram-positive, short, non-motile, pleomorphic rod. It grew well at 23°C in nutrient media containing a fermentable carbohydrate. Gelatin and starch were slowly hydrolyzed by C. insidiosum. Indole and hydrogen sulfide were not produced in organic media, and nitrates were not reduced by this species. Acid was produced from dextrose, lactose, sucrose, galactose and glycerine by the wilt bacterium. One culture of C. insidiosum isolated from alfalfa in Wisconsin, produced more acid than other cultures.

Blue, granular, extracellular deposits present in cultures of C. insidiosum are peculiar to this microorganism. Continued selection of the bluest colonies on a yeast agar, as reported by Starr (57), eventually resulted in the formation of colonies that were almost black. He further outlined methods by which the pigment was isolated and identified. Fulkerson (13) isolated white pigmented strains of C. insidiosum along with blue pigmented types.

Fulkerson (12, 14) has demonstrated differential pathogenesis among strains of C. insidiosum. One strain was found to be less virulent than another on a particular clone originating from DuPuits alfalfa. The two strains inoculated individually into a second clone caused equal levels of disease severity. At the alfalfa conference in Saskatoon,

Canada in 1960 Fulkerson reported that strains of C. insidiosum differing in virulence to a single clone of alfalfa were isolated from the same portion of a field. These strains maintained their relative virulence in agar culture.

Cook and Katznelson (3) isolated two different bacteriophages of C. insidiosum from soil. Phage P Ci 20 lysed 18 cultures of C. insidiosum while phage P Ci 2 lysed only one culture. Another culture of C. insidiosum was resistant to both phages. On the basis of bacteriophage specificity, therefore, three distinct strains of C. insidiosum were defined.

Rosenthal and Cox (54) have reported that C. insidiosum and Corynebacterium michiganense (Smith) Jensen are serologically homogeneous species distinct from other species of Corynebacterium. Some of the strains of the two species examined by them contained the same antigens.

SCOPE OF PRESENT STUDY

The present study was concerned with two phases; namely, (1) the persistence of C. insidiosum in soil and (2) the variability of this pathogen. Most emphasis was placed on the second phase of this study. Shortly after the second phase was initiated it became limited to the investigation of the antagonism against closely related cultures shown by a supposedly distinct strain of the pathogen.

MATERIALS AND METHODS

Source of Cultures

Jensen (29) has referred to all members of the genus Corynebacterium as the coryneform bacteria. The term coryneform as it appears in this thesis has been limited to the phytopathogenic bacteria of this genus.

The sources of cultures of C. insidiosum were as follows: C. insidiosum 4 was kindly supplied by Dr. W. H. Burkholder of Cornell University in 1957. Cultures 16 and 20 of C. insidiosum were isolated from infected alfalfa roots obtained in the Brookings area in 1955. Cultures 50, 83, and 96 of C. insidiosum were isolated in 1956 from infected roots originating from Tulare and Henry, South Dakota. The remaining isolates of wilt bacteria originated from infected roots collected near Newell, South Dakota in 1957. Of these, cultures 141, 143, 145, 158 and 163 were antagonistic types of the species, and culture 166 was a non-antagonistic one. Much use was made of culture L of C. insidiosum which was gratefully received from Dr. M. W. Cormack of the Science Service Laboratory at Lethbridge, Canada. Two other strains of C. insidiosum, culture N (antigenic strain AB) and culture N53 (antigenic strain BC) were kindly supplied by Dr. C. D. Cox of Department of Microbiology, University of South Dakota at Vermillion.

Culture 9 of C. michiganense was received from Dr. Burkholder in 1957. Three other cultures of C. michiganense were obtained from Dr. Cox's collection, namely cultures 102 (antigenic strain AB), culture 4450 (antigenic strain B) and culture 7429 (antigenic strain AB).

The sources of all strains from this latter collection has been previously outlined by Rosenthal and Cox (54).

Cultures of Corynebacterium sepedonicum (Spieckermann and Kotthoff) Skaptason and Burkholder and Corynebacterium poinsettiae (Starr and Pirone) were received from Dr. Burkholder. A culture of Corynebacterium flaccumfaciens (Hedges) Dowson var. aurantiacum Schuster and Christiansen was received through the kind auspices of Dr. M. L. Schuster, Department of Plant Pathology, University of Nebraska, Lincoln.

The permission to use non-coryneform bacterial cultures from collection of the Department of Bacteriology at South Dakota State College was kindly granted by Dr. H. E. Calkins. They were: Serratia marcescens Bizio, Escherichia coli (Migula) Castellani and Chalmers, Aerobacter aerogenes (Kruse) Beijerinck, Pseudomonas fluorescens (Migula), Sarcina lutea (Schroeter) and Bacillus cereus (Frankland and Frankland).

Medium Employed

An agar medium previously employed by Burkholder (2) for the growth of C. sepedonicum, designated as "Burkholder's agar," was used in this study. It was modified slightly by using dibasic potassium phosphate in place of the dibasic sodium salt, and using one-tenth as much asparagine as recommended. This medium was used for stock cultures of all of the microorganisms employed in this study and for studies in the persistence of C. insidiosum.

Plant Inoculations and Disease Assessment

The virulence of the different cultures of C. insidiosum was

tested by inoculating Rhizoma alfalfa. Alfalfa plants were started from seed in five-inch pots containing three parts of non-sterilized soil and one part of sand. Plants were thinned to 10 to 12 plants per pot. When the seedlings were at least 3½ months old, they were inoculated with turbid suspensions obtained from actively-dividing cells on agar-slant cultures of C. insidiosum. One hundred milliliters of a suspension was used to inoculate the plants in one pot by the root-ball soak method of Cormack et al. (5). In some instances single plants were lifted from the field and inoculated by the bare-root soak method described by Cormack et al. (5) which was similar to the immersion methods of other workers (8, 58, 60). Each culture was inoculated into duplicate pots of alfalfa which were then placed in a greenhouse maintained at 20° to 24°C. Supplementary lighting was provided during the winter months, and the plants were kept well-watered and the tops were trimmed every 2 to 3 weeks.

A minimum time of 3 months was allowed to pass after inoculation before the plants were removed from the soil and rated for disease. One plant of alfalfa which possessed a forked root did not exhibit bacterial wilt symptoms until almost 10 months after it had been inoculated with wilt bacteria. Generally the plants were left intact until top symptoms were evident.

To rate the extent of infection and damage in wilt-infected alfalfa plants, the system developed by Cormack et al. (5) was employed.

Incubation

Unless otherwise designated, all cultures of the microorganisms

employed in this study, and cells of C. insidiosum seeded into soil, were incubated at room temperature. Every endeavor was made to maintain the air temperature at 20 to 25°C. For certain critical experiments, warm-air incubators were kept in a cold room and the temperature in the room and incubators was carefully controlled. Cultures of C. insidiosum were incubated from 5 to 7 days depending upon their physiological vigor. When plate counts were performed on soils seeded with C. insidiosum, a two-week incubation period was allowed in order that colonies of this species would be of adequate size and pigmentation density.

Cultures of C. michiganense, C. flaccumfaciens var. aurantiacum, C. poinsettiae and the non-coryneform bacterial species required an incubation time of only 2 days, but this again depended upon the state of the stock cultures. Streptomycete-like microorganisms and cultures of C. sepedonicum required fairly long incubations. Some cultures of the latter had to be incubated for 2 weeks or longer until growth was apparent.

Persistence Studies of C. insidiosum in Soil

Two soils were employed for the bulk of the studies in persistence of C. insidiosum. They were collected from the Plant Pathology plots of the Experiment Station in Brookings. In addition, soils of different hydrogen-ion concentrations were collected from different areas of South Dakota. The pH of the soils was determined with a Model H2 Beckman pH meter using soil brought to the saturation point with boiled and cooled distilled water. The soils were moistened to various field moisture

capacities (F.C.) with distilled water or with very turbid suspensions of C. insidiosum.

To determine field-moisture capacities soil was thoroughly crushed and screened and placed in a thin layer on a greenhouse bench and air-dried. A 200 gram sample of this soil was placed in a 250 milliliter graduate cylinder, and distilled water was added until the column of soil was wet except for a dry plug at the bottom. This preparation was left standing overnight. Next morning the upper portion of the wet column was discarded and the central portion was retained for moisture determinations. The percentage of moisture in the center was considered to be the moisture content at field capacity. From the percentage of water in soil in the air-dry state and at field capacity, the amount of water that was needed in a given weight of soil to bring it to the required field capacity was easily determined. To estimate the moisture in a soil near the wilting point, air-dry soil was placed in a humid chamber for 5 to 7 days. When this period had expired moisture determinations were carried out.

One-hundred gram lots of soil were moistened to the required field capacity, allowed to stand for 2 to 4 hours and then thoroughly mixed with a heavy steel rod to distribute the bacterial cells.

In one experiment washed and sterilized sand was moistened several times with a very concentrated suspension of C. insidiosum and left to dry in circulating air after each addition. Ten grams of this dry sand inoculum was added to 100 grams of air-dry soil to ascertain how long the alfalfa-wilt bacterium could persist under such conditions.

Flasks of soil were sterilized by autoclaving at 250°F for 1 hour on 3 consecutive days. Between autoclavings the flasks were incubated at room temperature.

Immediately after sterilized or non-sterilized soil was inoculated with cells of C. insidiosum, 20 gram portions of seeded soil were serially diluted and plate counts on the alfalfa-wilt bacterium were carried out by conventional techniques. Acti-dione was added to the plating medium at a concentration of 250 p.p.m. to inhibit the growth of soil fungi which normally overgrew the plates. Three Petri dishes were employed for any one dilution of a soil sample and the counts were averaged. Bacterial counts of soils were reported on a per gram basis, and all other counts on a per milliliter basis. Two or more replicate flasks of soil were examined in this manner. The initial cellular concentrations of C. insidiosum in the seeded soils were high enough that at a 10^{-6} or higher dilution the indigenous soil microorganisms were diluted out and the wilt bacterial colonies were easily discernible. A number of days after the soils were seeded, wilt bacteria, if they were still present, formed colonies on agar which could be detected at dilutions of 10^{-5} or higher. The characteristic blue to purplish colonies this pathogen formed on agar could be readily distinguished from other microorganisms present in the soil. A greenish-blue pigmented, gram-negative bacterium was sometimes encountered in soil platings; however, it was readily differentiated from colonies of C. insidiosum.

Studies of an Antagonistic Variant of C. insidiosum

Morphological, Cultural and Serological Techniques

Coryneform and other bacterial cultures were stained by the Burke and Kopeloff-Beerman modifications of the gram stain as outlined in the Manual of Microbiological Methods (44).

To demonstrate whether or not various sugars were fermented by C. insidiosum, sterile nutrient broth was added aseptically to each autoclaved carbohydrate and transferred into cotton-stoppered test tubes. Individual tubes were tested up to 3 weeks after inoculation by means of phenol-red indicator and a model H2 Beckman glass electrode pH meter.

Tests for indole and hydrogen sulfide production, nitrate reduction, gelatin and starch hydrolysis by cultures of C. insidiosum were carried out according to methods given in the Manual of Microbiological Methods (44). C. insidiosum was grown in one percent tryptone broth and Kovacs test was applied for indole production. Lead acetate agar was used to determine the production of hydrogen sulfide by different cultures. The reduction of nitrates to nitrites was examined in nitrate agar making use of sulfanilic acid-α-naphthylamine indicator. To test for gelatin liquefaction, Frazier's method as modified by Smith was employed. Plates of nutrient agar containing 0.2 per cent soluble starch were inoculated with C. insidiosum, and at various times Lugol's iodine was flooded around the developing giant colonies to examine for starch hydrolysis.

The preparation of antigens and antisera for tube agglutination tests was carried out according to methods given by Rosenthal and Cox (54). Burkholder's agar was used in place of "eugonagar" to prepare

the antigens.

Methods for Evaluating Antagonism by Cells and Filtrates of Corynebacteria

Sensitive cultures of C. insidiosum or C. michiganense were subcultured twice on Burkholder agar slants. These rapidly-dividing cells of the second subculture were suspended in a small volume of sterile distilled water. One milliliter of a 10^{-2} dilution of this suspension was added to 100 milliliters of melted agar cooled to 45°C. This seeded agar was gently mixed and poured immediately into six Petri dishes to as uniform a depth as possible. When the agar had solidified, actively-growing antagonistic cells of C. insidiosum or C. michiganense or other antagonistic microorganisms were spotted on the seeded plates in a uniform pattern. The plates were then incubated until clear zones of inhibition were evident around the giant colonies. The extent of inhibition by antagonistic cells was reported as "zone width" which is the difference between the diameter of the inhibitory zone and the diameter of the antagonistic giant colony.

Various antibiotics were tested against C. insidiosum by placing antibiotic sensitivity disks (Difco, medium concentration) on agar plates seeded with cells of the two species in the manner just outlined. As before they were incubated until clear zones of inhibition were produced. Inhibition was reported as the diameter of the inhibitory zone.

Filtrates from cultures of C. insidiosum and C. michiganense were tested against sensitive strains of the two species by either of two methods. In the first method melted and cooled agar seeded with test cultures was poured around sterile rubber stoppers in Petri dishes.

After the seeded agar solidified, the rubber stoppers were aseptically removed and the resulting depressions were filled with the filtrates. In the other method sterilized stainless steel penicylinders were gently warmed and carefully placed on the surface of seeded agar. The penicylinders were filled with the filtrates. The amount of inhibition produced by the inhibitory filtrates was designated as the diameter of the inhibitory zone. Two replicate penicylinders were employed for each test filtrate.

RESULTS

Persistence of Corynebacterium insidiosum in Soil

The soil, as a haven and place of operation for cells of C. insidiosum, is a particularly pertinent medium to the alfalfa plant for the reason that wilt bacteria are believed to infect the plant from it. For that reason any information concerning the various biological and physical factors which might affect the longevity of the bacterium in soil would be helpful in understanding disease incidence and control. The present study was aimed to determine how long the wilt bacterium would survive in soil. Preliminary results on this phase of the study have already been published by this author (43).

Persistence of Dried Cells on Glass Surfaces

At the start the capability of wilt bacteria to persist on glass surfaces was investigated because under such conditions an estimate would be obtained of the inherent capability of the cells to persist in a dry state freed from the protective influence of soil colloids. Jones and McCulloch (35) had already reported that such cells could not survive longer than 23 days at room temperature; however, they had not investigated the effect temperature might have on such survival. Accordingly, the present study was aimed in that direction, and for that purpose, one-milliliter amounts of aqueous suspensions of wilt bacteria prepared from agar slants were put into a number of dry, cotton-stoppered, 125 milliliter erlenmeyer flasks, and agitated on a rotary shaker until dry. After different periods of storage in the

dark at different temperatures, the cells in randomly selected flasks were re-suspended in five milliliters of sterile distilled water, serially diluted and plated. The numbers of viable cells remaining in these flasks after different periods of storage are recorded in Table I. The alfalfa wilt bacterium persisted much longer in this test than it did in Jones and McCulloch's experiment (35). The shortest period of persistence was over 2.5 months at room temperature (open to laboratory at 20 to 25°C) and the longest was over 7 months at -20°, 5°, 10°, 20 to 25° (enclosed in a desiccator with drierite) and 30°C. The best persistence after 7 months was at -20°C. The number of viable cells after 2.5 months was essentially the same as that after 1 month. In all cases there was a sharp initial decline in numbers of viable bacteria during the first month of storage. This was especially true of the exposed flasks held at room temperature. This suggests some relationship to the sharply fluctuating humidities of late summer at the time when this experiment was started. Death of cells occasioned by initial drying could account for diminutions in viable cell count at the other temperatures after one month's time.

Persistence of Wet Cells in Sterilized Soil

As a further preliminary step toward understanding the capability of the alfalfa wilt bacterium to survive in soil, wilt bacteria were added to sterilized soil to ascertain (a) the suitability of this medium for growth of the bacterium and (b) the ability of the bacterium to persist in the absence of interferring biotic influences. The few tests that were conducted with such soils, however, were designed

TABLE I. PERSISTENCE OF C. INSIDIOSUM L ON GLASS SURFACES AT DIFFERENT TEMPERATURES

Temperature (°C)	Plate Count/ml. After[a]		
	1 month	2.5 months	7 months
-20	6.2×10^6	4.5×10^6	8.4×10^5
5	4.5×10^6	3.4×10^6	4.2×10^2
10	4.4×10^6	-	2.3×10^2
Room temp.	2.0×10^3	1.9×10^3	0
Room temp. (desiccator)	5.0×10^5	5.0×10^5	4.0×10^3
30	1.8×10^5	1.1×10^5	9.9×10^1

[a]Initial suspension count/ml. = 1.4×10^9

primarily to determine persistence at high moisture levels. In the first test non-sterilized soil was moistened to 120 per cent and 180 per cent of field capacity and sterilized, and a small amount of inoculum added to each soil. The soils were plated at different periods after inoculation, and the wilt bacterial counts were assessed on them with results appearing in Table II. The numbers of viable wilt bacteria present in these soils remained relatively constant over 39 days of storage; nevertheless, increases were evident at the eighth day at both moisture levels, but more at the higher level. These increases were followed by a very slow decline to the 39th day.

In another test combined with one using non-sterilized soil to be reported later (Table VI), wilt bacteria added to sterilized soil at 120 per cent of field capacity declined steadily from an initial count of 6.2×10^7 cells per gram to near 3.0×10^5 cells per gram over a 76 day period. Possibly, the failure of the bacterial cells to grow in this test was due to the higher initial number of bacterial cells added to the soil, which caused the cells of the inoculum to affect each other adversely.

In still another test, combined with one using non-sterilized soil to be reported later (Table VIII), the high numbers of wilt bacteria added to sterilized soil at 120 per cent of field capacity declined from 1.2×10^9 cells per gram to 4.9×10^7 cells per gram after 66 days of storage out-of-doors during the winter.

Thus wilt bacteria were able to persist for long periods in sterilized soil at high moistures.

TABLE II. PERSISTENCE OF C. INSIDIOSUM 4 IN STERILIZED SOIL[a] AT TWO MOISTURE LEVELS

Time After Soil Inoculation (days)	Plate Count[b]/gm. at	
	120% of F.C.	180% of F.C.
0	1.0×10^6	8.5×10^5
8	7.7×10^6	2.4×10^8
17	3.2×10^6	9.7×10^6
39	1.9×10^5	2.2×10^6

[a]Soil collected in Aug., 1957; expt. started Dec., 1957.

[b]Each count is the average count of 3 replicate flasks.

Persistence in Non-sterilized Soil

The introduction of the alfalfa wilt bacterium into non-sterilized soil contained in flasks presents this bacterium with a complex of microbiological influences that it normally encounters in nature. To assess the extent of these influences in a preliminary way the following three experiments were conducted.

In the first of these, wilt bacteria were added to quadruplicate flasks of soil moistened to 120 per cent of field capacity and stored at room temperature. The numbers of wilt bacteria fell from an initial count of 1.5×10^9 cells per gram to 3.0×10^6 cells per gram within 4 days, and to 0 cells per gram by 7 days time. In a companion test to the one just outlined, equal volumes of a 10^{-1} dilution of the soil and a 10^{-1} dilution of a suspension of the wilt bacterium incorporated together were incubated at room temperature. The numbers of wilt bacteria in this suspension fell from 2.8×10^8 cells per milliliter to 0 cells per milliliter after being incubated for 4 days.

The detection of wilt bacteria in non-sterilized soil was based upon the recognition of wilt bacterial colonies in agar platings of such soils. Consequently, if only a few viable wilt bacteria were present in soil, they could be obscured by soil microorganisms in platings of the lower soil dilutions. Such a low concentration of viable wilt bacterial cells in soil could, nevertheless, still be capable of infecting susceptible alfalfa plants and thereby be detected. Accordingly, four different cultures of the wilt bacterium were added singly to four different lots of the same soil moistened to 120 per cent

of field capacity. At the start and at weekly intervals thereafter, representative samples of these four seeded soils were put into suspension and used to inoculate Rhizoma alfalfa by the root-ball soak method. As may be seen by the results in Tables III and IV, the cells of all four of the wilt bacterial cultures were virulent upon being added to soil. Only one of the cultures was infective in non-sterilized soil after being held there for 7 days. At the 14th day and after, no virulent bacteria could be detected by this method. Therefore, the plant infection method essentially was no better than the plate method for detection of the wilt bacterium in non-sterilized soil.

(a) Biotic Factors Influencing Persistence of Wet Cells in Non-sterilized Soil

Many microorganisms not indigenous to non-sterilized soil upon being introduced into it may be parasitized or ingested by members of the soil microflora or microfauna, or acted upon indirectly by toxins and other inhibitory substances elaborated by these soil microorganisms. Also by indirect means, soil microorganisms may compete successfully for the food supply of the non-indigenous microorganisms. These direct and indirect biotic influences exerted by soil microorganisms are in turn dependent upon such environmental factors as moisture, temperature, soil reaction, and aeration, and upon kinds and relative abundance of these soil microorganisms which vary from field to field according to the cultural practices employed. It is not known how these biotic factors contribute to the decline of C. insidiosum when cells of this phytopathogen are added to soil; consequently, the following four

TABLE III. AVERAGE DISEASE RATINGS OF ALFALFA PLANTS INOCULATED WITH A NON-STERILIZED SOIL[a] PREVIOUSLY SEEDED WITH CULTURES OF C. INSIDIOSUM

Cultures Used to Seed Soil	Average Disease Ratings[b]				
	Days After Soil[c] Seeded with C. insidiosum				
	0	7	14	21	28
C. insidiosum 50	1.0	0.0	0.0	0.0	0.0
C. insidiosum 96	0.4	0.1	0.0	0.0	0.0
C. insidiosum 141	1.7	0.0	0.0	0.0	0.0
C. insidiosum 145	3.3	0.2	0.0	0.0	0.0

[a]Soil collected Oct., 1959; experiment started Jan., 1960.
[b]Each disease rating is the average of 4 replicate pots with 0 indicating no infection and 5.0 indicating maximum infection.
[c]Soil moistened to 120 per cent of F.C.

TABLE IV. PERCENTAGE OF SEVERELY DISEASED PLANTS INOCULATED WITH A NON-STERILIZED SOIL[a] PREVIOUSLY SEEDED WITH CULTURES OF *C. INSIDIOSUM*

Cultures Used to Seed Soil	Average Percentage[b] of Severely Diseased Plants[c] Days After Soil[d] Seeded with *C. insidiosum*				
	0	7	14	21	28
C. insidiosum 50	16.8	0.0	0.0	0.0	0.0
C. insidiosum 96	3.8	0.0	0.0	0.0	0.0
C. insidiosum 141	30.6	0.0	0.0	0.0	0.0
C. insidiosum 145	52.8	2.8	0.0	0.0	0.0

[a]Soil collected Oct., 1959; experiment started Jan., 1960.
[b]Each rating is the average rating of 4 replicate pots.
[c]Each plant with a disease rating of greater than 3.0.
[d]Soil moistened to 120 per cent of F.C.

experiments were performed to appraise these contributions.

In the first experiment, three different lots of the same sterilized soil moistened to 100 per cent of field capacity were prepared. To the first lot, cells of the wilt bacterium were added, to the second cells of the wilt bacterium along with a small portion of a diluted suspension of a soil streptomycete antagonistic to C. insidiosum, and to the third cells of the wilt bacterium and a small portion of a diluted suspension of a non-sterilized soil. Non-sterilized soil moistened to 100 per cent of field capacity and seeded with cells of the wilt bacterium served as a control. The results of this experiment appear in Table V. Five days after these four soils were seeded, the viable count of the wilt bacterium in the sterilized soil and the two sterilized soils originally inoculated with non-coryneform microorganisms had not dropped greatly from the initial counts. In the non-sterilized soil a noticeable reduction in wilt bacterial numbers had taken place. During the first 5 days the non-coryneform populations had increased appreciably in the two sterilized soils originally inoculated with these microorganisms. At the end of 20 days, the wilt bacteria had disappeared from all of the soils associated with non-coryneform soil microorganisms. In the sterilized soil alone the wilt bacterium still persisted in high numbers.

The biotic effects of the soil microflora on the longevity of the wilt bacterium were further demonstrated by a second experiment with two non-sterilized soils obtained from the Plant Pathology plots of the Experiment Station in Brookings. The first of these soils was collected

TABLE V. PERSISTENCE OF C. INSIDIOSUM 20 IN STERILIZED SOIL AT 100 PER CENT OF FIELD CAPACITY IN ASSOCIATION WITH NON-STERILIZED SOIL[a] AND A SOIL ANTAGONIST[b]

Soil Treatment	Plate Count[c]/gm. for C. insidiosum 20 at			Non-coryneform Organisms at		
	0 days	5 days	20 days	0 days	5 days	20 days
Sterilized Soil	1.2×10^9	5.0×10^8	3.1×10^7	0	0	0
Sterilized Soil and Soil Antagonist	1.8×10^9	5.9×10^8	0	4.4×10^3	8.0×10^5	1.8×10^7
Sterilized Soil and Non-sterilized Soil	1.6×10^9	3.3×10^8	0	4.9×10^2	3.5×10^7	6.1×10^7
Non-sterilized Soil	1.6×10^9	2.5×10^7	0	3.1×10^6	3.8×10^6	4.3×10^6

[a]Soil collected in Oct., 1959; experiment started Feb., 1960.
[b]A soil streptomycete antagonistic to C. insidiosum.
[c]Each count is the average count of 2 replicate flasks.

in August of 1957 and stored indoors in the air-dry state for 19 months, while the second soil was collected in October of 1959 and had only been indoors 2 weeks when this experiment was started. It was thought that certain microorganisms normally present in soil, and antagonistic to the wilt bacterium, might be gradually inactivated under conditions of prolonged dry storage. Both of the soils moistened to 120 per cent of field capacity were seeded with cells of C. insidiosum, and as a control the 1959 soil was sterilized, moistened to 120 per cent of field capacity and also seeded with cells of the wilt bacterium. From the results appearing in Table VI, one can see that the alfalfa wilt bacterium persisted no longer than 5 days in the 1959 non-sterilized soil while it persisted longer than 20 days in the 1957 non-sterilized soil. In the sterilized soil the wilt bacterium very slowly declined over a 76 day period. Since C. insidiosum did not survive any longer in the 1957 non-sterilized soil when it was first collected than it did in the 1959 non-sterilized soil used in this experiment, undoubtedly non-sterilized soil stored indoors in a dry state for relatively long periods of time affected certain components of the soil microflora. In platings of lower dilutions of the 1959 seeded and non-sterilized soil, a large number of streptomycete-like colonies were observed which were not evident in similar platings of the 1957 seeded and non-sterilized soil; furthermore, many of the streptomycetes isolated from the 1959 soil were highly inhibitory to the alfalfa wilt bacterium as determined in agar seedings of this phytopathogen. This experiment and the preceding one gave ample evidence that the overall soil microflora was responsible

TABLE VI. PERSISTENCE OF C. INSIDIOSUM 20 IN STERILIZED AND NON-STERILIZED SOILS[a] COLLECTED IN 1957 AND 1959

Time After Soil[b] Inoculation (days)	Plate Count[c]/gm. in:		
	Non-sterilized Soil		
	1957	1959	1959 Sterilized Soil
0	1.7×10^9	1.4×10^9	6.2×10^7
5	3.7×10^7	0	2.9×10^7
10	1.0×10^6	0	3.6×10^6
20	1.0×10^6	0	4.8×10^5
76	-	-	3.0×10^5

[a]Soil collected Aug., 1957 and Oct., 1959; experiment started Nov., 1959.
[b]Soil moistened to 120 per cent of F.C. and incubated at room temperature.
[c]Each count is the average count of 3 replicate flasks.

for the decline of C. insidiosum in soil, and that soil streptomycetes probably contributed measurably to this decline.

Since alfalfa wilt bacteria disappeared rapidly from the field soil collected from the experiment station in Brookings, six different soils were obtained from widely separated points representing different soil textures and reactions, which might be expected to have different microfloras. To determine what effects such a variable group of soils might have on the wilt bacterium, these soils moistened to 100 per cent of field capacity were seeded with cells of C. insidiosum. These soils were plated at the time of seeding and 5 days later. From the results for this experiment shown in Table VII, one can observe that the wilt bacterium persisted longer in some soils than others regardless of their texture or pH. The only explanation which accounts for the relative effectiveness or ineffectiveness of these soils in eliminating the wilt bacterium is that some of the soils, by design or by accident, had a more effective microflora than other soils, and as a consequence wilt bacteria could not persist in them for too long a time.

In view of the dissimilarities noted in the capability of the wilt bacterium to persist in different soils, attention was directed to the effects that plant growth might have on the persistence of the wilt bacterium in soil. Diachun and Valleau (6, 7) have found that certain phytopathogenic bacteria could overwinter in soil in association with wheat roots. To find if the wilt bacterium could persist in soil in such associations, the following experiment was carried out. Seedlings of Marquis, Lee, Rushmore and Selkirk varieties of wheat were

TABLE VII. PERSISTENCE OF C. INSIDIOSUM 20 IN SOILS[a] AT DIFFERENT HYDROGEN-ION CONCENTRATIONS MOISTENED TO 100 PER CENT OF F.C.

Soil[b] pH	Soil Texture	Plate Count[c]/gm. at: 0 days	5 days
5.6	clay	1.8×10^9	7.0×10^5
5.9	silt loam	5.7×10^8	0
6.2	loamy sand	1.3×10^9	0
7.0	silt loam	1.4×10^9	1.0×10^6
7.5	clay	2.7×10^9	0
7.6	clay	2.3×10^9	5.0×10^6

[a]Soil collected Oct., 1959; experiment started May, 1960.
[b]Seeded soil incubated at 20°C.
[c]Each count is the average count of 3 replicate flasks.

grown in pots of non-sterilized soil in the greenhouse. When the seedlings reached the three-leaf stage of development, depressions were made in the soil around each plant by pushing a heavy iron wire into the soil in such a way as to sever some of the fibrous roots of each plant. These depressions were filled with very turbid suspensions of the wilt bacterium, and the remainder of the soil was then thoroughly wetted with the suspensions. Thirty-seven days after these inoculations, 24 plants of each variety were removed from the soil with some of the adhering soil particles and the roots of these were crushed and put into suspension. Agar platings of serial dilutions of these suspensions did not reveal the presence of any wilt bacteria in association with the wheat roots or the surrounding soil. As to how long the wilt bacterium persisted in this soil during the 37 day interim is not known; nevertheless, the wheat roots did not prolong the phytopathogen's persistence under greenhouse conditions.

(b) Persistence of Cells in Non-Sterilized Soil as Influenced by Moisture and Temperature

Under favorable conditions of soil reaction and oxygen and food supply, the numbers and overall physiological activity of soil microorganisms increases with rise in soil temperature and moisture to an optimum level. If alfalfa wilt bacteria were added to non-sterilized soil under conditions that were optimal to soil microorganisms antagonistic to this phytopathogen, one might expect that with a rise in soil temperature and moisture there would be a more rapid decline of the wilt bacterium than in soil in which the temperature and moisture were

lowered. To evaluate these effects three separate experiments were performed.

The first experiment was designed to ascertain persistence under cold winter conditions. Sterilized and non-sterilized soil moistened to 120 per cent of field capacity were seeded with cells of the wilt bacterium. An assessment was made of the numbers of wilt bacteria in these soils. They were then left to overwinter in flasks stored in depressions chopped out of frozen field soil. The flasks were shielded from the sun's rays by two heavy metal lids. Soil temperatures were not recorded during the time these flasks of seeded soil were in the field. From climatological data, however, the mean of the average of the maximum and minimum daily air temperatures for the first 43 days the flasks were held in the field was found to be -11.9°C, and for the last 23 days was 5.1°C. On 3 different days toward the end of the time the flasks were in the field, the average of the maximum and minimum daily air temperatures rose above 10°C; however, none of these temperatures went above 13.6°C. If the air temperatures on an average basis did not rise much beyond 5°C during the experiment, one can safely assume that the soil temperature was close to 0°C. Counts were made on the two soils upon removal from the field. One can conclude from the data given in Table VIII that the numbers of wilt bacteria were not appreciably different in either of the two soils at either the beginning or the end of the experiment. Possibly at these low temperatures the antagonistic influences of the soil microflora were not operative. The decrease in numbers of viable wilt bacteria over the

TABLE VIII. PERSISTENCE OF C. INSIDIOSUM 20 IN FLASKS OF STERILIZED AND NON-STERILIZED SOIL[a] MOISTENED TO 120 PER CENT OF F.C. AND ALLOWED TO OVERWINTER IN THE FIELD

Time After Soil Inoculation (days)	Plate Count[b]/gm. in:	
	Sterilized Soil	Non-sterilized Soil
0	1.2×10^9	1.6×10^9
66	4.9×10^7	2.1×10^7

[a] Soil collected Oct., 1959; experiment started Feb. 13, 1960.
[b] Each count is the average count of 3 replicate flasks.

66 day period in both of the soils resulted, presumably, from mechanical injury to the cells, or normal declines that occur in every population of bacterial cells.

The second experiment was designed to assess further the effect of soil moisture and temperature on persistence of the alfalfa wilt bacterium in soil. The soil used was one that was collected in 1957 and stored in the laboratory for 19 months. In this experiment the soil moistened to 50 per cent and 100 per cent of field capacity was seeded with wilt bacteria and stored at either 10°C or 20°C. The results recorded in Table IX and Figure 1 show that the numbers of viable wilt bacteria in this soil kept at 10°C at either of the two moisture levels were not altered substantially over 12 days time; however at the higher moisture level there was a noticeable decline during this period. Wilt bacteria were more persistent in soil moistened to 50 per cent of field capacity and stored at 20°C than in soil at the same temperature moistened to 100 per cent of field capacity. At the latter moisture level a very decided reduction in wilt bacterial numbers occurred during the 12 day holding period.

In the third experiment the soil used was similar to the one employed in the preceding experiment except that it had been stored for only 4.5 months in the laboratory. This soil adjusted to 13.0 per cent, 53.5 per cent and 100 per cent of field capacity was seeded with wilt bacteria and stored at -20°, 10° and 20°C. The numbers of viable wilt bacteria remaining in these soils after different periods of storage are recorded in Table X and Figure 2. The viable wilt bacterial numbers

TABLE IX. PERSISTENCE OF C. INSIDIOSUM 20 IN 1957 NON-STERILIZED SOIL[a] AT DIFFERENT MOISTURES AND TEMPERATURES

Time After Soil Inoculation (days)	Plate Count[b]/gm. in Soil at:			
	50% of F.C.		100% of F.C.	
	10°C	20°C	10°C	20°C
0	880,000,000	880,000,000	2,100,000,000	2,100,000,000
2	490,000,000	690,000,000	2,000,000,000	1,800,000,000
4	670,000,000	450,000,000	1,400,000,000	770,000,000
6	530,000,000	61,000,000	1,400,000,000	230,000,000
8	610,000,000	57,000,000	1,400,000,000	87,000,000
12	610,000,000	39,000,000	1,100,000,000	3,000,000

[a]Soil collected Aug. 1957; experiment started Mar. 9, 1959.
[b]Each count is the average count of 2 replicate flasks.

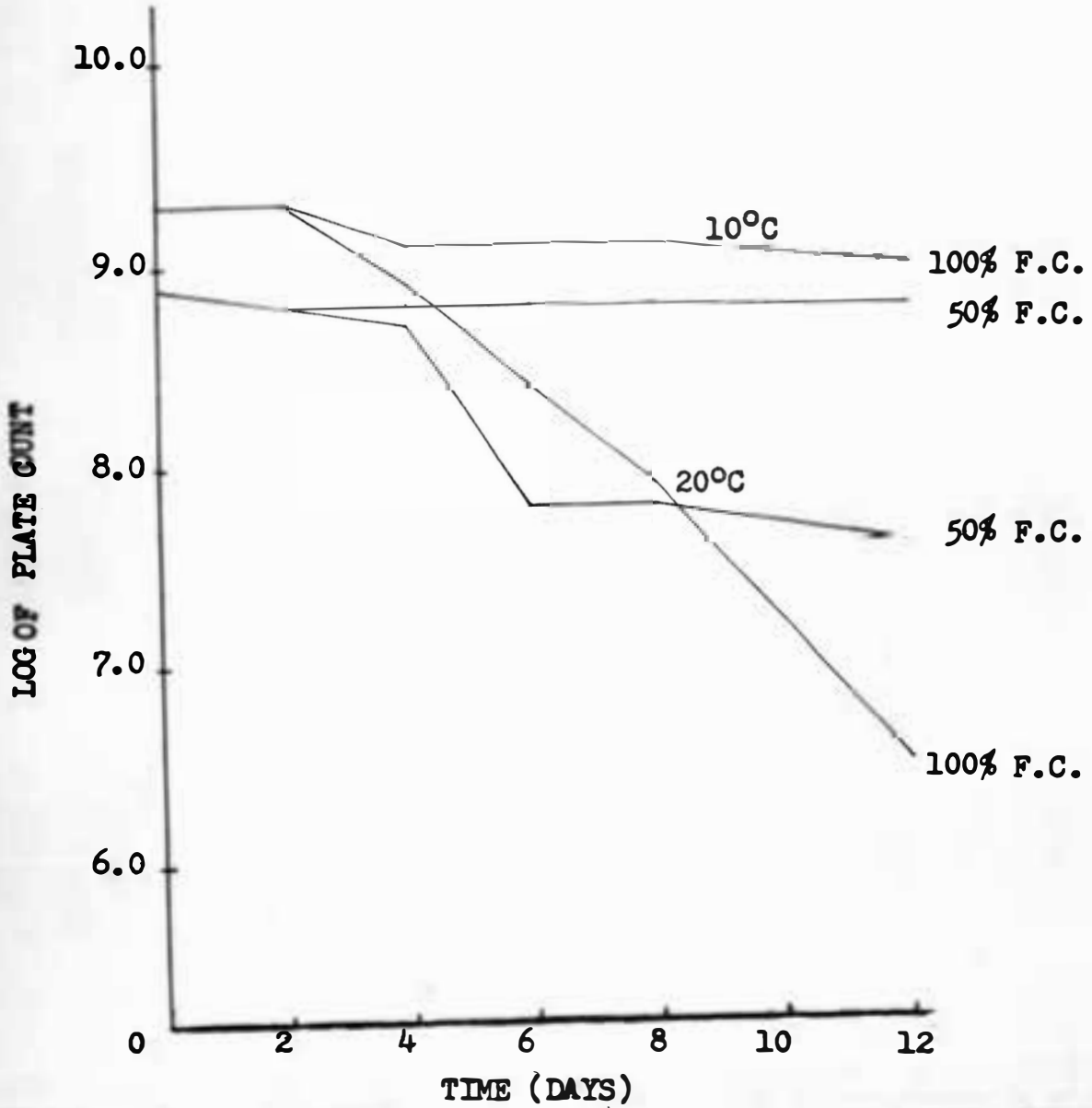


Figure 1. Effect of Moisture and Temperature on the Count of C. insidiosum 20 Heavily Seeded into Non-sterilized Soil

TABLE X. PERSISTENCE OF C. INSIDIOSUM 20 IN 1959 NON-STERILIZED SOIL[a] AT DIFFERENT MOISTURES AND TEMPERATURES

Time After Soil Inoculation (days)	Plate Count[b]/gm. in Soil at:								
	13% of F.C.[c]			53.5%[d] of F.C.			100% of F.C.		
	-20°C	10°C	20°C	-20°C	10°C	20°C	-20°C	10°C	20°C
0	8.7×10^7	8.7×10^7	8.7×10^7	7.8×10^8	7.8×10^8	7.8×10^8	1.0×10^9	1.0×10^9	1.0×10^9
5	-	8.2×10^7	4.8×10^7	-	5.7×10^8	7.2×10^7	-	2.9×10^8	3.0×10^6
10	-	8.2×10^7	3.8×10^7	-	3.6×10^8	2.0×10^7	-	1.0×10^8	0
15	-	8.2×10^7	3.8×10^7	-	7.1×10^7	2.0×10^6	-	2.5×10^6	0
20	8.7×10^7	7.9×10^7	2.8×10^7	7.8×10^8	3.9×10^7	0	7.2×10^8	5.0×10^5	0
169	7.5×10^7	4.1×10^7	6.0×10^6	4.1×10^8	0	0	5.1×10^8	0	0

[a]Soil collected Oct., 1959; experiment started Apr. 16, 1960.
[b]Each count is the average count of 2 replicate flasks.
[c]Air-dry soil.
[d]Wilt point at 30.3 per cent of field capacity.

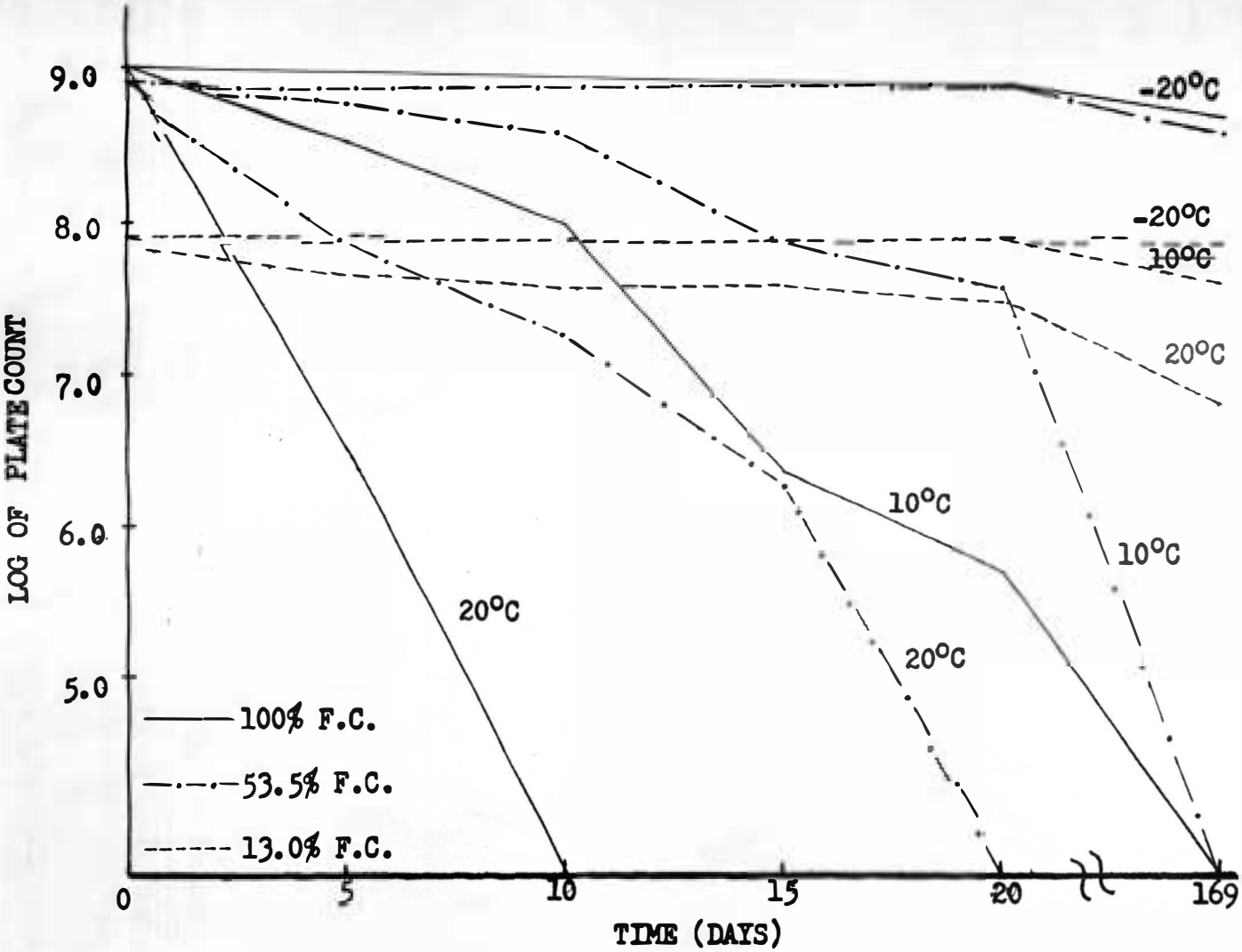


Figure 2. Persistence of C. insidiosum 20 in 1959 Non-sterilized Brookings' Soil at Different Moisture Levels and Temperatures

were not altered to any great degree in soils maintained at -20°C at either of the three moisture levels over 169 days time. In soils maintained at 10°C a measurable reduction in numbers of viable wilt bacteria occurred in soil moistened to 100 per cent of field capacity while such reductions were less pronounced at the other two moisture levels over 20 days time. At the end of 169 days, however, wilt bacteria were persistent only in the air-dry soil. At 20°C viable wilt bacteria were not recovered from soils moistened to 100 per cent or 53.5 per cent of field capacity after 20 days time. In air-dry soil held at this temperature wilt bacteria were still persistent at the end of 169 days. Consequently, as the soil temperature and moisture levels were increased in this non-sterilized soil, the wilt bacterial population declined at a faster rate.

Persistence in Diseased Alfalfa Roots Retained in Moist Soil

Jones and McCulloch (35) found that C. insidiosum could survive for at least 5 months in hay while Cormack et al (5) determined that in infected and dried plants (presumably roots) wilt bacteria could survive for 3 years at room temperature. Peltier and Jensen (48) were unable to isolate viable wilt bacteria from infected plants left in the field overwinter. One can conclude, therefore, that wilt bacteria do not survive long in infected plants in association with the soil microflora. To determine how rapidly wilt bacteria are inactivated in infected plant material added to soil, roots of infected alfalfa from which wilt bacteria were isolated were placed in non-sterilized soil. The soil was kept well-watered and maintained at 20 to 25°C. One month

later the roots were dug up and found to be intact, but in an advanced state of decay. From the stelar portions of these unsound roots no wilt bacteria were isolated; however, a large number of non-coryneform microorganisms were present in platings of these roots. By the time infected roots had progressed to this state of deterioration the wilt bacteria had presumably been inactivated.

An Antagonistic Variant of Corynebacterium insidiosum

Late in 1957 agar platings of wilt-infected alfalfa roots from near Newell, South Dakota, yielded purplish-red, wilt-like bacterial colonies that were inhibitory to the growth of other wilt bacterial colonies developing in the same plate. Since the antagonistic colonies were new to C. insidiosum, the writer proceeded to learn whether or not they could be considered a strain of that species and, if so, to learn something of the production and properties of the antagonistic principle they elaborated. Preliminary results on this phase of the study have already been reported by the writer (42).

Physiological Characteristics of the Antagonistic Wilt-like Bacteria

Culturally on Burkholder's agar medium, all isolates of the antagonist yielded blue to purplish-red pigmented colonies that were butyrous, smooth, glistening, entire and raised. The cells were gram-positive, short, pleomorphic rods, as seen from nigrosin and gram stains. The colonies and cells were like those of authenticated C. insidiosum cultures possessed by the writer and also like those described for that species by Jones and McCulloch (35). The purplish-red pigmentation was

within the range of pigmentation ascribed to that species.

In tests of carbohydrate utilization, indole and hydrogen sulfide formation, nitrate reduction, starch hydrolysis and gelatin liquefaction, the antagonist proved similar if not identical to C. insidiosum. In the carbohydrate utilization test, conducted as outlined under "methods," one isolate of the antagonist was compared with three authentic cultures of C. insidiosum. From the differences in final pH evident in Table XI between the sugared and non-sugared media, one may judge that the antagonist fermented all but one of nine sugars fermented by C. insidiosum; namely, dextrose, sucrose, lactose, maltose, galactose, mannose, xylose and salacin. The one exception was mannitol, but this may not be unusual as Ramamurthi (52) reported his cultures of C. insidiosum to be incapable of fermenting the carbohydrate in an organic medium. Dulcitol, raffinose, melezitose, and sorbitol were not fermented by any of the cultures.

In the other test mentioned above, also conducted as outlined under "methods," two isolates of the antagonist were compared with six authentic cultures of C. insidiosum with results presented in Table XII. The two isolates of the antagonist were like the six cultures of C. insidiosum in hydrolyzing starch, in not reducing nitrates and in not producing indole or hydrogen sulfide; however, they were unlike the six in liquefying gelatin slightly. With regard to the last property, however, most investigators have reported gelatin liquefaction by C. insidiosum.

In sensitivity to antibiotics and other inhibitory compounds,

TABLE XI. CARBOHYDRATES FERMENTED BY STRAINS OF C. INSIDIOSUM AND THE ANTAGONIST

Strain	Acid Reaction[a] Produced in Tubes Containing													Check
	Dextrose	Sucrose	Lactose	Maltose	Galactose	Mannose	Mannitol	Xylose	Salacin	Dulcitol	Raffinose	Melezitose	Sorbitol	Nutr. broth
C. insidiosum L.	6.5	6.3	6.8	6.6	6.5	6.8	6.7	6.5	6.8	7.5	7.5	7.5	7.0	7.3
C. insidiosum 4	6.7	6.5	6.9	6.5	6.8	7.0	6.7	6.8	6.9	7.5	7.6	7.5	7.0	7.5
Antagonist 145	6.3	6.3	6.6	6.4	6.3	6.4	7.2	6.3	6.9	7.2	7.2	7.4	7.2	7.3
C. insidiosum 20	6.5	6.5	7.0	6.6	6.6	6.8	6.7	6.5	6.8	7.4	7.4	7.4	7.0	7.3
Uninoculated Control	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0

[a]pH measured with a Colman pH meter 16 days after broth was inoculated.

TABLE XII. PHYSIOLOGICAL REACTIONS OF C. INSIDIOSUM AND ANTAGONISTIC ISOLATES

Strain	Indole Produced	H_2S Produced	Nitrate Reduced	Starch Hydrolyzed	Gelatin Liquefied
C. insidiosum L	-	-	-	-	-
C. insidiosum 4	-	-	-	Sl. +	-
Antagonist 141	-	-	-	Sl. +	+
Antagonist 145	-	-	-	Sl. +	Sl. +
C. insidiosum 20	-	-	-	Sl. +	-
C. insidiosum 96	-	-	-	-	-
C. insidiosum N	-	-	-	Sl. +	-
C. insidiosum N53	-	-	-	Sl. +	-

the antagonist was also similar to if not identical with C. insidiosum. This similarity was observed from a test described under "methods" with 27 antibiotic substances against two isolates of the antagonist and six cultures of C. insidiosum. As may be seen from the diameters of the inhibitory zones produced and recorded in Table XIII, the sensitivities of the two isolates of the antagonist were similar to those of the six cultures of C. insidiosum to all of the inhibitory substances except para-amino-salicylic acid (PAS) and isonicotinic acid hydrazide (INH). With these two anti-bacterial agents a separation was possible among some of the cultures but not to the extent that the two cultures of the antagonist were separable from all six cultures of C. insidiosum.

Serologically also the antagonist proved similar to C. insidiosum. Homologous antisera were prepared as described under "methods" to one culture of the antagonist, to four of C. insidiosum and to one of C. michiganense, the latter being serologically homogeneous with C. insidiosum (54). These were used in cross agglutination tests with washed and steamed cells of all cultures with results shown in Table XIV. The antiserum against the antagonist agglutinated the cells of C. insidiosum and C. michiganense and the antisera homologous to these species agglutinated the cells of the antagonist. Although some of the titers in these cross-agglutination tests were not high, the results obtained demonstrated the likeness of the antagonist to C. insidiosum.

Pathologically also the antagonist proved like C. insidiosum in producing typical bacterial wilt symptoms in Rhizoma alfalfa. In an infection test, 14 antagonistic isolates from the one field near Newell,

TABLE XIII. SENSITIVITY OF STRAINS OF C. INSIDIOSUM AND THE ANTAGONIST TO VARIOUS ANTIBIOTICS AND INHIBITORY COMPOUNDS

Chemical Inhibitors	Zone diameter in agar[a] seeded with C. insidiosum culture						Antagonists	
	L	4	20	96	N	N53	141 & 145	
Elkosin	30	25	23	45	35	25	30	45
Gantrisin	34	35	40	50	30	40	40	50
Sulfadiazine	35	33	30	45	30	30	35	50
Sulfamerazine	29	25	25	40	35	20	23	35
Sulfathiazole	35	35	23	50	45	30	32	42
Thiosulfil	25	30	20	55	40	30	23	40
Triple sulfa	30	25	33	35	30	-	27	40
Furadantin	55	45	58	55	50	48	45	47
Furacin	51	40	50	45	47	41	38	42
Magnamycin	59	52	50	60	60	60	53	63
Neomycin	15	20	25	32	20	20	15	35
Novobiocin	70	60	65	70	65	60	80	60
Oleandomycin	45	40	27	70	50	37	25	45
Streptomycin	35	28	37	35	40	30	27	30
Viomycin	0	0	0	0	0	0	0	0
INH	0	0	20	12	0	0	14	20
PAS	0	0	20	0	0	0	0	0
Mandelamine	35	35	42	35	25	25	20	20
Aureomycin	65	60	65	70	66	60	70	68
Chloromycetin	45	42	55	60	48	40	40	50
Dihydrostreptomycin	30	30	33	33	33	28	35	40
Erythromycin	60	60	80	60	60	60	60	64
Penicillin	50	50	60	52	50	50	50	52
Polymyxin B	0	0	0	0	0	0	0	0
Terramycin	50	57	60	60	53	50	65	70
Tetracycline	55	50	65	65	60	50	62	70
Bacitracin	55	57	64	65	60	55	55	63

[a]Burkholder's agar

TABLE XIV. AGGLUTINATION TITERS[a] OF VARIOUS CORYNEBACTERIAL ANTISERA USING WASHED AND STEAMED ANTIGENS

Antigen	Antisera Homologous to: Strains of *C. insidiosum* L	N53	20	4	CM[b]9	Antagonist 145
C. insidiosum L	128	256	256	128	256	256
C. insidiosum N53	512	256	1024	512	1024	512
C. insidiosum 20	16	4	128	8	256	64
Antagonist 145	64	8	64	8	1024	128
C. insidiosum 4	256	128	128	128	512	256
C. michiganense 9	32	8	128	8	256	64

[a]Reciprocal of the highest serum dilution which effected agglutination.
[b]CM = *C. michiganense*.

a number of isolates of C. insidiosum obtained from diseased alfalfa from various parts of South Dakota, and other cultures of C. insidiosum were used to inoculate alfalfa by the root-ball soak method. The severity of disease symptoms incited by the antagonists did not differ materially from symptoms incited by the C. insidiosum isolates.

Thus, in all of these tests the antagonist proved similar to if not identical with C. insidiosum. However, proof that the antagonist could be considered a strain of C. insidiosum was provided by the following test. Four cultures of the antagonist and six authentic cultures of C. insidiosum were seeded separately into Burkholder's agar medium and each of the agar seedings was spot-planted with all 10 of the above cultures. As shown in Table XV the four antagonistic cultures inhibited the six authentic cultures, with none of the latter inhibiting any of the former. Neither the antagonist nor the authentic cultures were self-inhibitory. In a later test two antigenically different strains of C. insidiosum, cultures N and N53 with AB and BC antigenic designations, respectively, and culture 20 of C. insidiosum were found to be non-inhibitory to an authentic culture of C. insidiosum, but they were inhibited by the antagonist of that species. As the authentic cultures of C. insidiosum used in these tests were derived from widely divergent geographical areas of this continent and represented at least two serologically different strains, the antagonistic cultures thus may be considered a strain of this species, dividing the species into antagonists and non-antagonists.

Stability and Production of the Inhibitory Principle Elaborated by the Antagonistic Strain of C. insidiosum

TABLE XV. INHIBITION OF C. INSIDIOSUM BY ANTAGONISTIC CULTURES OF THAT SPECIES

Spotted Test Strains	Zone Width (mm.) in Agar[a] Seedings of Different Strains of C. insidiosum: Seeded Strains of C. insidiosum						Seeded Strains of Antagonist			
	4	L	16	83	96	166	143	145	158	163
C. insidiosum 4	0	0	0	0	0	0	0	0	0	0
C. insidiosum L	0	0	0	0	0	0	0	0	0	0
C. insidiosum 16	0	0	0	0	0	0	0	0	0	0
C. insidiosum 83	0	0	0	0	0	0	0	0	0	0
C. insidiosum 96	0	0	0	0	0	0	0	0	0	0
C. insidiosum 166	0	0	0	0	0	0	0	0	0	0
Antagonist 143	10	12	14	10	14	12	0	0	0	0
Antagonist 145	10	14	14	10	16	12	0	0	0	0
Antagonist 158	12	14	14	10	10	12	0	0	0	0
Antagonist 163	10	14	12	10	16	12	0	0	0	0

[a]Burkholder's agar

The inhibitory characteristic of the antagonistic strain of C. insidiosum was detected by spot planting cultures of that strain onto the surface of Burkholder's agar medium containing a heavy seeding of ordinary wilt bacteria. The procedure was similar to that widely used by others in detecting antagonists to various bacteria. The antagonism was expressed as a 10-18 mm. wide zone clear of growing seeded wilt bacteria in such a way that the zone was faintly clear within 3 to 4 days and strikingly clear within 7 days (Figure 3A). The width of the zone did not increase measurably from the fourth to seventh day. As most, if not all, of the seeded wilt bacteria in the zone did not grow, the action of the antagonistic principle on these cells was deemed bacteriostatic rather than bactericidal.

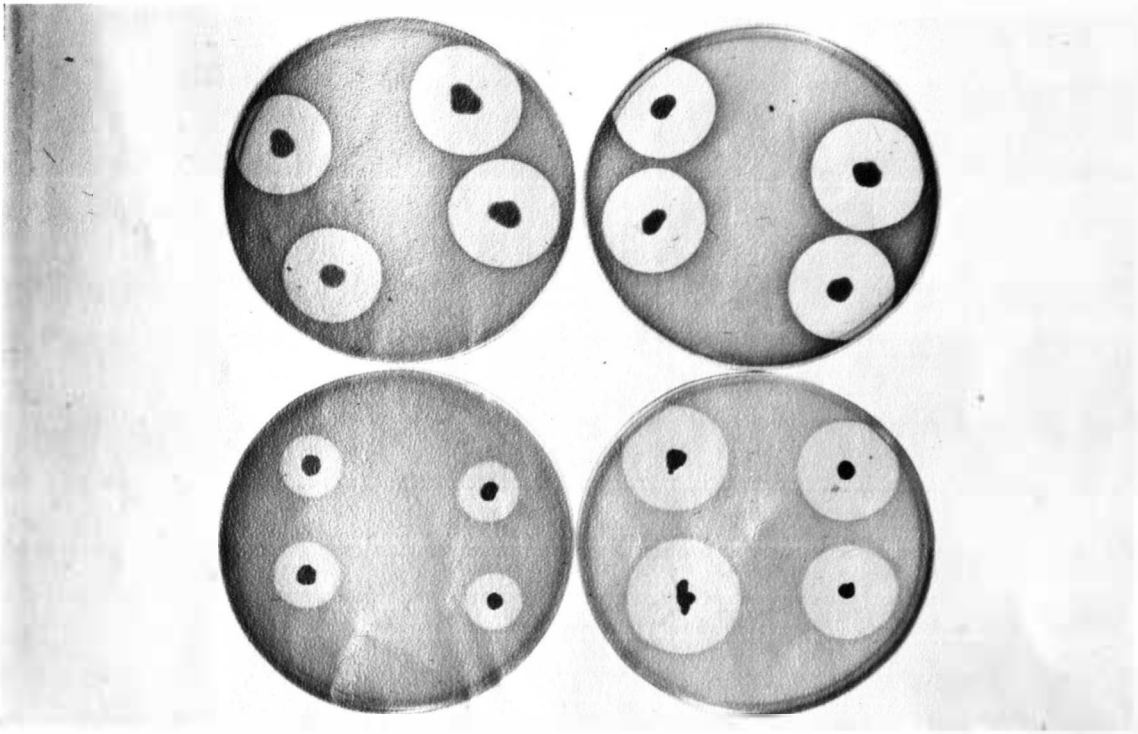
To determine whether or not the antagonistic property of the antagonistic strain was a uniform, stable characteristic of the cultures on hand, the following series of tests was conducted. In the first of these, highly diluted cell suspensions of antagonistic cultures 141 and 145 were plated on Burkholder's medium and from each of them 70 colonies, presumably originating from single cells, were subcultured and tested against wilt bacterial cultures 4 and L. The entire lot of 140 subcultures proved inhibitory, thus demonstrating the purity of antagonistic cultures 141 and 145 for this characteristic.

In the second test, the same two antagonistic cultures and two ordinary cultures, 50 and 96, were inoculated separately by the root-ball soak method into Rhizoma alfalfa grown in the greenhouse. When wilt symptoms appeared on the aerial foliage of these plants two months

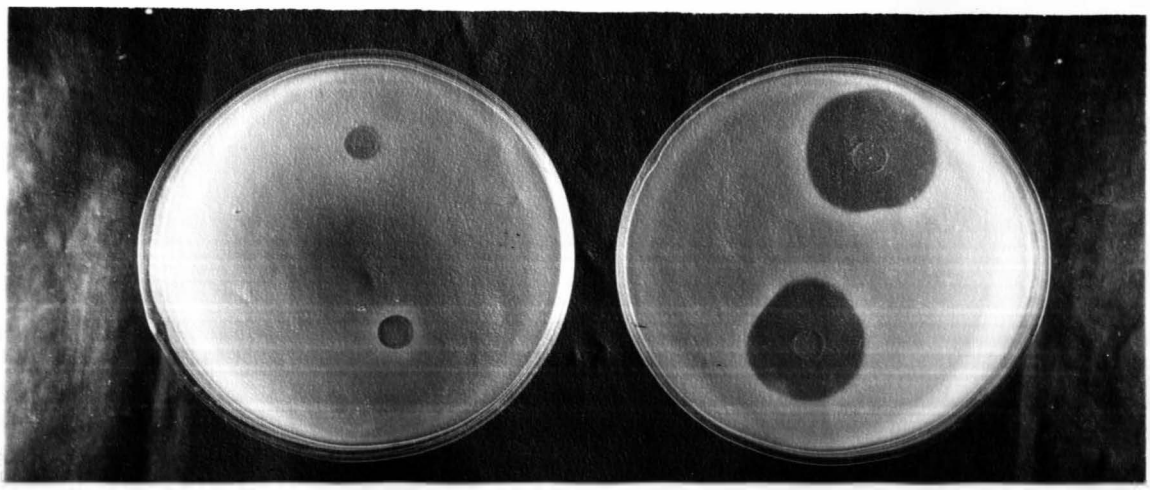
Figure 3. Inhibition Zones Produced by Cells and Agar-culture Filtrates of an Antagonistic Strain of C. insidiosum on Agar Seeded with a Non-antagonistic Strain of the Same Species

A. Spot-planted Cells on Seeded Agar; Lower Left Burkholder's Agar, Remainder Peptone-deficient Burkholder's Agar

B. Spot-planted Filtrates on Seeded Agar; Left Unheated Filtrate, Right Filtrate Heated at 100°C for 1 Minute



A



B

Figure 3.

later, root platings yielded wilt bacterial colonies from which were established 79 random cultures of antagonist 141, 69 of antagonist 145, 24 of non-antagonist 96 and 26 of non-antagonist 50. In all instances the cultures proved inhibitory or non-inhibitory to agar seedings of ordinary C. insidiosum culture 96 according to whether they originated from plants inoculated with inhibitory or non-inhibitory cultures. In this instance also one may conclude that antagonistic cultures 141 and 145 were pure for that characteristic and that the characteristic was stable on passage of the bacteria through alfalfa.

In the next test one of the above antagonistic isolates (culture 145) and one of the non-antagonistic cultures (culture 96) were inoculated separately into two arms of a forked tap root of Rhizoma alfalfa to observe the stability and competitiveness of the antagonist when faced with a non-antagonist in the internal environment of an alfalfa root. The root was that of a mature plant lifted from the field with aerial foliage attached. The tips of the two arms were cut off about three inches below the joint and both arms were then simultaneously immersed to a depth of 1 inch for 2 hours in aqueous suspensions of the respective cultures. Thereupon, the plant was removed from the suspensions, trimmed of aerial foliage and transplanted into a large pot of soil in the greenhouse. When wilt symptoms appeared on the foliage nearly 10 months later, agar platings of each of the root arms and of the root above the fork yielded bacterial wilt colonies, random representations of which were cultured. Subcultures of these were then assayed for antagonistic and non-antagonistic properties by spotting

them on seedings of a non-antagonistic culture (culture L) in Burkholder's agar medium. As may be seen from the results presented in Table XVI, the antagonistic and non-antagonistic bacteria were both present not only in the portions of the root above the fork, but also in both arms of the fork. The proportion of antagonists to non-antagonists was about 1:1 in the first arm of the fork, about 4:1 in the second and about 1.5:1 in the region above the fork. As the identity of the arms inoculated with the two cultures was lost, no conclusions can be drawn from the differences in these proportions as to the passage of the two bacteria from one arm of the fork to the other. Similarly, as the two arms of the fork were in the same pot of soil, no inferences can be made as to how the antagonistic and non-antagonistic wilt bacteria passed from one arm of the fork to the other. Nevertheless, the presence of the two kinds of wilt bacteria in the same portion of the root indicates that the two were compatible, as may be judged from the original isolation of the two kinds of wilt bacteria from alfalfa roots near Newell, South Dakota.

The stability of the inhibitory property of the antagonist in the forked root may be determined from the following comparison of zone widths produced on Burkholder's agar medium. The inhibitory zone widths produced by 55 antagonistic cultures derived from the forked root ranged from as little as 2 mm. to as much as 20 mm., with most widths being 14 to 16 mm., inclusive. The average zone width was 13.8 mm. with a standard deviation of 3.16 mm. and a coefficient of variation of 22.8 per cent. In contrast 72 inhibitory zone widths produced from a stock culture of the same antagonist ranged from 5 to 17 mm., with a mean of 11.9 mm., a

TABLE XVI. INHIBITION OF C. INSIDIOSUM L BY WILT BACTERIAL CULTURES ISOLATED FROM A FORKED ALFALFA ROOT ORIGINALLY INOCULATED WITH NON-ANTAGONISTIC[a] AND ANTAGONISTIC STRAINS OF C. INSIDIOSUM

Isolates and Their Inhibitory Action	Isolations From:		
	First Fork	Second Fork	Below Crown
Total no. of random isolates	58	20	20
No. of antagonistic isolates	28	16	12
Percentage of antagonistic isolates	48	80	60
Average zone width on Burkholder's agar	13.8	13.2	15.0

[a]Plant with forked root originally inoculated with C. insidiosum 96 and C. insidiosum 145, a non-antagonistic and antagonistic strain respectively.

standard deviation of 1.9 mm. and a coefficient of variation of 16.0 per cent. Passage of the antagonist through the alfalfa root, therefore, appeared to enhance the variability of the inhibitory property so that some were extremely weak in that property and others strong. No explanation at the moment can be offered to account for this variability.

The variation in inhibitory intensity among antagonistic isolates noted in the preceding experiments prompted a series of studies of some nutritional and physical factors that might influence the expression of this intensity. In one of the first of such studies, Burkholder's agar medium was modified in various ways in two separate tests by deleting different components from it or by substituting others for them. The antagonistic culture 145 was evaluated against the non-antagonistic culture L. The width of the inhibitory zones produced in each of these tests is recorded in Tables XVII and XVIII. Also recorded in these tables are the average diameters of the spot-planted antagonistic colonies developing in these tests. The results in both tables may be discussed together in terms of the components present or lacking in modifications of that medium.

A modified Burkholder's medium containing only phosphate, sodium chloride, citrate, asparagine and dextrose supported only a very slight growth of the antagonist and of the seeded non-antagonist with no formation of an inhibition zone. Addition of 0.5 per cent peptone to this modification of Burkholder's medium yielded a markedly larger inhibitory zone than that produced in Burkholder's complete medium, which contained both peptone and potato extract, but the zone was indistinct because of

TABLE XVII. THE EFFECTS OF YEAST EXTRACT AND PEPTONE ON INHIBITION OF C. INSIDIOSUM L BY C. INSIDIOSUM 145

Medium No.	Concentration[a] of Ingredients in Different Media[b]								Average Zone Width[c] (mm.)	Average Diameter of Antagonistic Colony (mm.)
	Peptone	K_2HPO_4	N_aCl	Citrate	Asparagine	Dextrose	Potato extract	Yeast extract		
1	0	0.2	0.2	0.1	0.06	0.6	Ab.[d]	0	0.0	no growth
2	0.5	0.2	0.2	0.1	0.06	0.6	Ab.	0	17.0 (indistinct)	4.9
3	0	0.2	0.2	0.1	0.06	0.6	Pr.[e]	0	19.7	4.9
4	0.5	0.2	0.2	0.1	0.06	0.6	Pr.	0	10.8	4.9
5	1.0	0.2	0.2	0.1	0.06	0.6	Pr.	0	6.4	5.1
6	1.5	0.2	0.2	0.1	0.06	0.6	Pr.	0	4.0	6.0
7	2.0	0.2	0.2	0.1	0.06	0.6	Pr.	0	1.0	7.0
8	0	0.2	0.2	0.1	0.06	0.6	Ab.	0.5	20.4	7.2
9	0	0.2	0.2	0.1	0.06	0.6	Ab.	1.0	17.0	8.2
10	0	0.2	0.2	0.1	0.06	0.6	Ab.	1.5	17.0	9.4
11	0	0.2	0.2	0.1	0.06	0.6	Ab.	2.4	16.2	8.0

[a]In per cent.
[b]All media contained 1.2% agar.
[c]Average of 24 replicate zones.
[d]Ab. = Absent
[e]Pr. = Present

$L.S.D._{0.05} = 0.70$ $L.S.D._{0.01} = 0.91$

TABLE XVIII. INHIBITION OF C. INSIDIOSUM L BY C. INSIDIOSUM 145 ON MODIFIED BURKHOLDER'S AGAR

Medium No.	Concentration[a] of Ingredients in Different Media								Average Zone Width[b] (mm.)	Average Diameter of Antagonistic Colony (mm.)
	Peptone	K_2HPO_4	NaCl	Citrate	Asparagine	Dextrose	Potato Extract	Agar		
1	0.5	0.2	0.2	0.1	0.06	0.6	Pr.[c]	1.2	13.3	5.5
2	0	0.2	0.2	0.1	0.06	0.6	Pr.	1.2	18.5	4.9
3	0.5	0	0.2	0.1	0.06	0.6	Pr.	1.2	11.5	5.3
4	0.5	0.2	0	0.1	0.06	0.6	Pr.	1.2	11.5	5.2
5	0.5	0.2	0.2	0	0.06	0.6	Pr.	1.2	10.8	5.7
6	0.5	0.2	0.2	0.1	0	0.6	Pr.	1.2	11.9	5.5
7	0.5	0.2	0.2	0.1	0.06	0	Pr.	1.2	10.3	6.2
8	0.5	0	0	0	0.06	0.6	Pr.	1.2	5.1	5.0
9	0.5	0	0	0	0.06	0	Pr.	1.2	0.0	3.8

[a] In per cent.
[b] Average of 24 replicate zones.
[c] Pr. = Present.

$L.S.D._{0.05} = 1.02$ $L.S.D._{0.01} = 1.34$

retarded growth of the non-antagonist and the antagonist in that medium. Addition of potato extract instead of peptone to the same modification of Burkholder's medium yielded a clear zone of nearly the same size as with peptone. Thus with respect to these two ingredients, potato extract seemed necessary for production of clear inhibition zones which peptone alone was unable to produce. The two together in the modified Burkholder's medium yielded smaller inhibition zones than either of them did alone.

The presence of higher than normal concentrations of peptone in Burkholder's complete medium resulted in a marked reduction in size of inhibition zones, such that the size of the zone was inversely proportional to the concentration of peptone in the medium. Extrapolating from this relationship, one might reason, therefore, that the presence of the peptone in Burkholder's complete medium suppressed the size of the inhibition zone from that obtainable from peptone-deficient Burkholder's agar.

Yeast extract in a 0.5 per cent concentration substituted for both peptone and potato extract in Burkholder's medium resulted in an inhibition zone of a size comparable to that produced by a similar medium containing potato extract and no peptone. Progressively higher concentrations of the extract resulted in smaller inhibition zones, but the reductions in their size were very much less than with peptone. Twice normal concentrations of potato extract in peptone-deficient Burkholder's medium, incidentally, have yielded the same size inhibition zones as a normal concentration of the extract.

In the presence of both peptone and potato extract, deletions of phosphate, NaCl, citrate, asparagine, or dextrose slightly reduced the

size of the inhibition zone while deletions of the three salts and dextrose yielded no inhibition zone even though the spot-planted antagonist developed into a moderate sized colony.

Thus from these tests one may conclude that potato extract and yeast extract provided some factor (or factors) needed for the fullest expression of inhibition by the antagonist, and that the peptone lacked that factor and even contained an inhibitor that suppressed the size of the inhibition zone. From the colony sizes of the antagonist appearing in these tests and recorded in the tables one may also draw the conclusion that these factors had little if anything to do with the growth of the antagonist. Also they apparently had little if anything to do with the growth of the non-antagonist as this bacterium, like the antagonist, grew equally, or nearly equally, as well on Burkholder's complete and Burkholder's peptone-deficient media, as shown in Table XIX, from agar platings of the two bacteria in these media.

In another study the concentration of the non-antagonistic cells seeded into Burkholder's agar was tested as a possible source of variation of inhibitory zone widths. The same cultures of the antagonist and non-antagonist were used as above. As may be seen from the data in Table XX, the zone width was slightly increased as the concentration of the cells seeded into the medium was reduced by a factor of one hundred. The extent of this increase, however, was slight and thus concentration of seeded non-antagonistic cells in the medium had little effect on the expression of zone widths.

In a further study, the effect of the physiologic condition of

TABLE XIX. PLATE COUNTS OF SUSPENSIONS OF C. INSIDIOSUM L AND C. INSIDIOSUM 145 ON BURKHOLDER'S AND PEPTONE-DEFICIENT BURKHOLDER'S AGAR

Culture	Plate Count[a]/ml. on:	
	Burkholder's Agar	Peptone-deficient Burkh. Agar
C. insidiosum L (N^b)	1.4×10^{10}	1.2×10^{10}
C. insidiosum 145 (A^c)	1.0×10^{10}	3.1×10^{9}

[a]Each count is the average count of 4 replicate plates.
[b]Non-antagonistic strain.
[c]Antagonistic strain.

TABLE XX. THE EFFECT OF CONCENTRATION OF CELLS OF C. INSIDIOSUM L IN AGAR SEEDING ON THEIR INHIBITION BY C. INSIDIOSUM 145

Dilution[a] of Suspension of C. insidiosum L	Zone Width[b] (mm.)
10^{-1}	10.4
10^{-2}	11.0
10^{-3}	12.8

[a]One ml. of a dilution per 100 ml. of Burkholder's agar.
[b]Each width is the average width of 24 replicate zones.

$L.S.D._{0.05} = 1.06$ $L.S.D._{0.01} = 1.41$

stored cultures of the antagonist was tested for production of inhibitory zone widths. Cultures of the above antagonist stored at 5°C for 3.5, 4.5, 6.0 and 7.0 months were spot-planted directly onto peptone-deficient Burkholder's agar medium seeded with the above non-antagonistic culture. At the same time a four-day-old, freshly transferred culture maintained in the laboratory was spot-planted on the same medium. From the results presented in Table XXI, one may see that the four-day-old freshly transferred culture yielded the largest zones while all of the stored cultures spot-planted directly on the medium produced the smallest zones. The zone widths yielded by the latter cultures, however, varied erratically in relation to the age of those cultures. As a supplement to that test, other stored cultures of varying age were transferred onto fresh Burkholder agar slants and after two transfers the fresh cultures were spot-planted onto peptone-deficient Burkholder's agar medium seeded with the same non-antagonist. As may be seen from the results of that test presented in Table XXII, the inhibitory intensity of all stored cultures was restored to the same level and equal to that of freshly maintained cultures. Thus from the results of these tests, which correspond to other experiences of the writer, the antagonistic cultures maintained their inhibitory properties in cold storage but such cultures had to be put into an active state of growth by subculturing them at least once to obtain maximum inhibitory expression.

In still a further study, the effect of agar concentration in peptone-deficient Burkholder's agar medium was tested for production of inhibitory zone widths by the same antagonist against the same non-antagonist.

TABLE XXI. THE EFFECT OF AGE OF C. INSIDIOSUM 145 CULTURES ON THEIR INHIBITORY ACTION AGAINST C. INSIDIOSUM L

Age[a] of Culture (months)	Zone Width[b] (mm.)
0.1	20.6
3.5	2.9
4.5	5.4[c]
6.0	1.1
7.0	1.5

[a]Removed from refrigerator on Oct. 18, 1960 and spotted on seedings on the same day.

[b]Average of 8 replicate zones.

[c]Halo around inhibitory zone 1.8 mm. in width.

TABLE XXII. INHIBITION OF C. INSIDIOSUM L BY THE SECOND SUBCULTURE OF FOUR REFRIGERATED CULTURES OF C. INSIDIOSUM 145 OF VARYING AGE

Date[a] of Culture on Removal from Refrigerator	Zone Width[b] (mm.)
May 28, 1959	22.0
July 1, 1959	21.4
July 11, 1959	21.6
August 20, 1959	21.5

[a] Removed from refrigerator on Sept. 23; spotted on seeding Oct. 3.
[b] Average of 24 replicate zones.

For this purpose agar concentrations ranging from 0.8 to 3.0 per cent were provided in peptone-deficient Burkholder's agar medium with results appearing in Table XXIII. In this test the inhibitory zone widths proved linearly related to the logarithm of the agar concentration, but the range of these widths was narrow indicating that slight variations in normal agar concentration in the medium would have but little effect on the zone widths expected.

In a final study the effect of incubation temperature on zone width expression was tested on Burkholder's complete medium using the same antagonist and non-antagonist as in the above. For this purpose the inoculated agar plates were held at different temperatures with results appearing in Table XXIV. As is evident from this table, zones of inhibition appeared in plates held at 10°, 20° and 25°C but they did not appear in plates held at 4° to 5°C. The largest zones appeared at 20°C, which was the usual temperature maintained in the laboratory, followed by slightly smaller zones at 25°C. The range of the zone widths at these temperatures, however, was such that very little variation in zone width might be expected from fluctuating temperatures in the laboratory.

Nature of the Inhibitory Principle Elaborated by the Antagonistic Strain of C. insidiosum

From the foregoing fact that the antagonistic strain of C. insidiosum produced a wide inhibitory zone against non-antagonistic cultures of C. insidiosum, one may draw the self-evident conclusion that the inhibitory principle is probably an organic material diffusible in agar and, therefore, water-soluble. To learn something more about its

TABLE XXIII. THE EFFECT OF AGAR CONCENTRATION ON INHIBITION OF C. INSIDIOSUM L BY C. INSIDIOSUM 145

Agar Concentration (%)	Zone Width[a] (mm.)
0.8	21.9
1.0	21.4
1.2	20.7
1.5	19.6
2.0	19.0
2.5	18.4
3.0	17.5

[a]Average of 24 replicate zones.

TABLE XXIV. THE EFFECT OF INCUBATION TEMPERATURE ON INHIBITION[a] OF C. INSIDIOSUM L BY C. INSIDIOSUM 145

Temperature (°C)	Zone Width[b] (mm.)
4-5[c]	0
10	10
20	16
25	14

[a]On Burkholder's agar.
[b]Average of 10 replicate zones.
[c]Incubated for 1 month.

nature, an attempt was made first to obtain the inhibitory material free of antagonistic cells. In the first such attempt cultures of the antagonist alone or in combination with non-antagonists were grown in Burkholder's medium free of agar in stationary or shake culture. No inhibitory filtrates from such cultures were obtained when the cultures were passed through Seitz or ultra-fine, fritted-glass filters. In this as in all other tests, as outlined under methods, the sterile filtrates were spotted in Petri plates in regular penicillin cups (penicylinders) or in small depressions made by rubber stoppers surrounded with freshly poured peptone-deficient Burkholder's agar medium seeded heavily with a non-antagonistic culture of C. insidiosum.

In the second attempt inhibitory zones produced on Burkholder's agar medium were cut out, crushed and centrifuged, and the supernatant passed through ultra-fine, fritted-glass filters. This filtrate proved inhibitory as did similar filtrates in subsequent tests where the antagonist was grown alone on thin layers of Burkholder's agar medium. A control filtrate prepared from sterile Burkholder's agar in the same way as above proved non-inhibitory. Such agar cultures of the antagonist were the source of inhibitory material used in all studies of the nature of the inhibitory principle. These tests included the response of the inhibitor to ultrafiltration, centrifugation, desiccation, high temperatures, aeration, high temperatures at different pH values, and to organic solvents.

(1) Ultrafiltration

Extracts from agar cultures of C. insidiosum 145 were filtered

through a Seitz filter and a fritted-glass filter. The inhibitory principle produced by this strain did not pass through the Seitz filter but did pass through the fritted filter. Presumably, the electrical charge on the Seitz filter was such that the inhibitor was adsorbed on the filter pad.

(2) Centrifugation

Agar cultures of C. insidiosum 145 were crushed and centrifuged at 3,000 r.p.m. (1450G) and at 15,000 r.p.m. (17,000G). The supernatants were filtered through fritted-glass filters. Both filtrates were equally inhibitory to C. insidiosum L. High speed centrifugation, therefore, did not separate the inhibitor from the suspending fluid.

(3) Desiccation

An inhibitory filtrate of C. insidiosum 145 placed in a Petri dish with a clay-top cover was left to dry at room temperature. The dried material resuspended to one-half of its original volume with distilled water failed to inhibit C. insidiosum L.

(4) High Temperatures

When inhibitory filtrates of C. insidiosum 145 were heated and tested against C. insidiosum L, a most interesting phenomenon was observed (Figure 3B). As outlined in Table XXV, no change was observed in the inhibitory filtrate's behavior when it was held for 5 minutes at temperatures up to 50°C. Upon heating the filtrate at 60°C for 5 minutes, it was more inhibitory to C. insidiosum L than the unheated filtrate. The inhibitory effect of the filtrate heated at 70°C for 5

TABLE XXV. INHIBITION OF C. INSIDIOSUM L BY HEATED FILTRATES FROM AGAR CULTURES OF C. INSIDIOSUM 145

Heat Treatment[a] (°C)	Zone Diameter[b] (mm.)
Unheated	9
30	9
35	9
40	9
50	9
60	14
70	20
80	24
100	24

[a]Held at each temperature for 5 minutes.
[b]Each zone diameter is the average diameter of 2 replicate zones on peptone-deficient Burkholder's agar.

minutes was more than double that of the unheated filtrate. Even heating the filtrate of C. insidiosum 145 up to 100°C for 5 minutes almost tripled its effect over that of the unheated filtrate. Steaming this filtrate for 10 minutes caused the inhibitory effect to drop from that of the five-minute holding period as is shown in Table XXVI. Steaming for 20 minutes caused inhibition which was measurably greater than inhibition by the unheated filtrate; however, steaming for periods of 40 minutes or longer reduced the extent of inhibition to a level equal to or less than the extent of inhibition of the unheated filtrate. While steaming caused thermal activation of the inhibitory principle, autoclaving, as indicated in Table XXVII inactivated the inhibitor. Heated and unheated control filtrates prepared from Burkholder's agar were non-inhibitory to the non-antagonistic wilt bacterium.

(5) Aeration

The filtrates heated at the different temperatures shown in Table XXV were tested at the time of treatment, and 9 days after storage at refrigeration temperature. During refrigeration, these treated filtrates were stored in sterile, screw-cap test tubes. The data presented in Table XXVIII show that the inhibitory activity of the heated filtrates declined with aging under the indicated conditions. It was assumed that oxygen in the tube caused this decreased activity of the stored, heated filtrate. To test this hypothesis the experiment outlined in Table XXIX was carried out. Both heated and unheated filtrates were aerated for one hour with sterile air at atmospheric pressure. Aeration did not inactivate the inhibitory principle in the

TABLE XXVI. INHIBITION OF C. INSIDIOSUM L BY STEAMED AGAR CULTURE FILTRATES OF C. INSIDIOSUM 145

Heat Treatment	Zone Diameter[a] (mm.)
Unheated	10
80°C for 5 minutes	24
100°C for 1 minute	27
100°C for 2.5 minutes	25
100°C for 5 minutes	25
100°C for 10 minutes	19
100°C for 20 minutes	14
100°C for 40 minutes	11
100°C for 60 minutes	8

[a]Average of 2 replicate zones on peptone-deficient Burkholder's agar.

TABLE XXVII. INHIBITION OF C. INSIDIOSUM L BY AUTOCLAVED AGAR CULTURE FILTRATES OF C. INSIDIOSUM 145

Heat Treatment	Zone Diameter[a] (mm.)
Unheated	9
100°C for 1 minute	23
120°C for 1 minute	0
120°C for 5 minutes	0
120°C for 10 minutes	0
120°C for 15 minutes	0
120°C for 20 minutes	0

[a]Average of 2 replicate zones on peptone-deficient Burkholder's agar.

TABLE XXVIII. EFFECT OF REFRIGERATION OF HEATED-AGAR CULTURE FILTRATES OF C. INSIDIOSUM 145 ON THEIR INHIBITORY ACTION AGAINST C. INSIDIOSUM L

Heat Treatment[a] (°C)	Zone Diameter (mm.)[b]	
	Before Refrigeration	After Refrigeration[c]
Unheated	9	9
30	9	8
35	9	8
40	9	8
50	9	8
60	14	8
70	20	15

[a]Held at each temperature for 5 minutes.
[b]On peptone-deficient Burkholder's agar.
[c]Held at 5°C for 9 days.

TABLE XXIX. EFFECT OF AERATION OF HEATED AND UNHEATED AGAR CULTURE FILTRATES OF C. INSIDIOSUM 145 ON THEIR INHIBITORY ACTION AGAINST C. INSIDIOSUM L

Treatment of Filtrate	Zone Diameter[a] (mm.)
Unheated	9
Heated at 70°C for 5 minutes	20
Unheated and aerated for 1 hour	9
Unheated and aerated for 1 hour then heated at 70°C for 5 minutes	20
Heated at 80°C for 5 minutes then aerated for 1 hour	0

[a]On peptone-deficient Burkholder's agar.

unheated filtrate as was indicated by thermal activation of the unheated and aerated filtrate. When the filtrate was heated and then aerated, complete inactivation of the inhibitory material ensued.

Whatever alteration occurred in the configuration of the inhibitory principle upon heating and aerating is open to conjecture. An inhibitor which prevented the maximum expression of the antagonistic principle may have been inactivated upon heating, or possibly a degradation product more inhibitory than the original inhibitory material in the unheated filtrate formed upon heating and manifested itself under the proper conditions.

(6) High Temperatures at Different Hydrogen-ion Concentrations

The unheated agar culture filtrate from C. insidiosum 145 was adjusted to various pH levels with sterile sodium hydroxide or hydrochloric acid. After these acidic and basic filtrates were heated, they were adjusted as closely as possible to their original pH and tested for their effectiveness against C. insidiosum L. According to the data presented in Table XXX, the inhibitory principle was thermostabile and capable of being thermally activated in the pH range of 3.0 to 8.2. Above pH 8.2 and below pH 3.0 the inhibitor was inactivated by heating. One might reason that outside of the pH range 3.0-8.2 the inhibitor was not heat inactivated, and upon the return to this pH range of 3.0-8.2 it would be capable of being thermally activated; however, upon returning the filtrates from the higher or lower pH levels to a more neutral position and re-heating them, no such thermal activation occurred. Filtrates at a pH of 1.1 and 12.5 which were not heated and returned to

TABLE XXX. EFFECT OF HYDROGEN-ION CONCENTRATION ON THERMOSTABILITY OF INHIBITORY FILTRATES FROM AGAR CULTURES OF C. INSIDIOSUM 145

Treatment of Filtrate		Zone Diameter[c] (mm.) in Seedings of C. insidiosum L	
pH at which Heated	Final pH Adjustment of Filtrate	Filtrate Heated and Adjusted to Final pH	Filtrate Reheated After Final pH Adjustment
Heated[a] at pH 12.5	7.5	0	0
Unheated at pH 12.5	7.5	8	14
Heated at pH 12.0	7.5	0	0
Heated at pH 11.5	7.0	0	0
Heated at pH 11.1	7.2	0	0
Heated at pH 9.8	7.5	0	0
Unheated at pH 8.2[b]	8.2	9	20
Heated at pH 8.2	8.2	21	12
Heated at pH 6.7	8.3	25	16
Heated at pH 5.8	7.6	25	17
Heated at pH 4.3	7.8	24	15
Heated at pH 3.0	7.4	16	10
Heated at pH 2.0	7.5	0	0
Heated at pH 1.1	7.5	0	0
Unheated at pH 1.1	7.5	8	19

[a]Filtrates heated at 100°C for 2 minutes.
[b]pH of unadjusted filtrate.
[c]On peptone-deficient Burkholder's agar.

pH 7.5 were thermally activated by heating at 100°C for two minutes. Consequently, one could safely conclude that the inhibitor was inactivated by heat outside the limits of the stated pH range.

(7) Organic Solvents

The inhibitory agar-culture filtrate was treated with 2 to 3 times its volume of acetone or absolute ethyl alcohol. Precipitates formed and were left to settle in screw-cap test tubes in a refrigerator. After 3 to 4 hours, the supernatants were poured off as carefully as possible and the precipitates were resuspended to the original volume of the filtrate with a sterile salt solution (0.2 per cent NaCl, 0.2 per cent K_2HPO_4, 0.1 per cent tribasic sodium citrate and 0.06 per cent asparagine). The resuspended preparations were heated or left unheated and tested against C. insidiosum L. As shown in Table XXXI both acetone and ethanol precipitated out the inhibitory principle. In decanting the ethanol supernatant, some of the precipitate was lost which could account for the poorer inhibition by this preparation. The resuspended preparations were more effective after heating than before thus confirming that the inhibitor was carried down in the precipitate. Also, acetone not only precipitated out the inhibitor in the unheated filtrate but in the heated one as well.

Giant colonies of C. insidiosum 145 were allowed to develop for 7 to 9 days on Burkholder's agar medium. Only one colony was allowed to grow on each agar plate. Colonies were then exposed to chloroform vapor in a closed desiccator jar for different periods of time. At the end of the exposures the plates were covered with clay tops to allow

TABLE XXXI. PRECIPITATION OF THE INHIBITORY PRINCIPLE FROM AGAR-CULTURE FILTRATES OF C. INSIDIOSUM 145 BY TWO ORGANIC SOLVENTS

Filtrate Treatment	Zone Diameter[a] (mm.) in Seedings of C. insidiosum L
Unheated	14
Heated at 100°C for 1 minute	28
Acetone precipitate from unheated filtrate - unheated	17
Acetone precipitate from unheated filtrate - heated at 100°C for 1 minute	25
Acetone precipitate from filtrate heated at 100°C for 1 minute	24
Ethanol precipitate from unheated filtrate - unheated	10
Ethanol precipitate from unheated filtrate - heated at 100°C for 1 minute	14
Salt solution used to resuspend precipitates	0

[a]On peptone-deficient Burkholder's agar.

chloroform vapor to escape. Four hours later, 9 cm. sterile filter paper disks impregnated with a concentrated suspension of C. insidiosum L were used to imprint bacteria onto the surfaces of the agar plates containing the chloroform-treated giant colonies. Prior to this seeding, the viability of the treated giant colonies was checked by streaking them onto sterile Burkholder agar slants. The results in Table XXXII show that chloroform vapor killed the antagonistic cells, but did not inactivate the inhibitory principle. Larger zones of inhibition formed around giant colonies exposed for 6 hours to chloroform than around those exposed for 20 hours. The 20 hour exposure caused a substantial reduction in the activity of the inhibitory principle.

Specificity of the Inhibitory Principle Elaborated by the Antagonistic Strain of C. insidiosum

To elucidate further the nature of the inhibitory principle elaborated by the antagonistic strain of C. insidiosum, a series of tests were performed to learn of the specificity or non-specificity of the action of the inhibitor on other bacteria.

In the first of these tests a heated (one minute at 100°C) and an unheated inhibitory filtrate of culture 145 of the antagonistic strain of C. insidiosum were spotted on peptone-deficient Burkholder's agar medium seeded separately with a number of different coryneform and non-coryneform bacterial species. Both heated and unheated filtrates markedly inhibited three cultures (L, N and N53) of the non-antagonistic strain of C. insidiosum (Table XXXIII) while they did not inhibit single cultures of C. flavefaciens var. aurantiacum, C.

TABLE XXXII. INHIBITION OF C. INSIDIOSUM L BY GIANT COLONIES OF C. INSIDIOSUM 145 EXPOSED FOR DIFFERENT TIMES TO AN ATMOSPHERE OF CHLOROFORM

Time Exposed to Chloroform (hours)	Zone Diameter[a] (mm.)	Viability of Giant Colonies After Exposure to Chloroform
0	43	+
6	42	-
20	35	-

[a]Each zone diameter is the average diameter of 4 replicate zones.

TABLE XXXIII. EFFECT OF AGAR CULTURE FILTRATES OF C. INSIDIOSUM 145 ON CORYNEFORM AND NON-CORYNEFORM BACTERIAL SPECIES

Agar Seeded With	Zone Diameter (mm.) Produced by Filtrate of C. insidiosum 145	
	Unheated	Heated[a]
C. insidiosum L	14	26
C. insidiosum N	18	32
C. insidiosum N53	19	33
C. poinsettiae 2	0	0
C. sepedonicum 16	0	0
C. flaccumfaciens var. aurantiacum	0	0
S. marcescens	0	0
E. coli	0	0
A. aerogenes	0	0
P. fluorescens	0	0
B. cereus	0	0
S. lutea	0	0

[a]Heated 100°C for 1 minute.

poinsettiae, C. sepedonicum, Serratia marcescens, Escherichia coli, Aerobacter aerogenes, Pseudomonas fluorescens, Bacillus cereus and Sarcina lutea. The inhibitor in this test thus acted only on a member of its own species. Colonies of the antagonistic strain of C. insidiosum, however, later proved weakly inhibitory to C. sepedonicum.

In the next test, two cultures of the antagonistic strain and two of the non-antagonistic strain of C. insidiosum were spot-planted on Burkholder's agar medium seeded with four cultures of C. michiganense representing two serologically different strains of this latter species. As is evident from the results presented in Table XXXIV, the two cultures of the antagonistic strain of C. insidiosum did not inhibit two of the four cultures of C. michiganense while they did inhibit one slightly and the remaining one markedly. On the other hand, the two cultures of the non-antagonistic strain of C. insidiosum were essentially alike in that one did not inhibit any of the four C. michiganense cultures while the other only slightly inhibited all four. Thus in this test the action of the antagonistic strain of C. insidiosum was non-specific.

To explore this cross-inhibition to C. michiganense further, three tests were conducted. The first of these was concerned with ascertaining whether or not cultures of C. michiganense would inhibit cultures of the antagonistic strain and of the non-antagonistic strain of C. insidiosum, and whether or not they would inhibit one another. Accordingly four cultures of C. michiganense were spot-planted on Burkholder's agar medium seeded with the same four cultures and with

TABLE XXXIV. INHIBITION OF STRAINS OF C. MICHIGANENSE BY STRAINS OF C. INSIDIOSUM

Spot Planted Strains	Zone Width (mm.) in Agar[a] Seeded With C. michiganense Strains			
	9	4450	7429	102
C. insidiosum L (N[b])	1	1	1	1
C. insidiosum 96 (N)	0	0	0	0
C. insidiosum 141 (A[c])	0	0	2	10
C. insidiosum 145 (A)	0	0	2	10

[a]Burkholder's agar.
[b]Non-antagonistic strain.
[c]Antagonistic strain.

three cultures of C. insidiosum, of which one was an antagonistic strain and the other two non-antagonistic strains. As may be seen from the zone widths recorded in Table XXXV, two of the four C. michiganense cultures inhibited nearly equally well the one culture of the antagonistic strain and the two of the non-antagonistic strain of C. insidiosum. The other two cultures of C. michiganense markedly inhibited only one culture of the non-antagonistic strain of C. insidiosum. The four cultures of C. michiganense did not inhibit themselves or one another, except that cultures 9 and 4450 mutually inhibited one another slightly. Thus from this test one might conclude that the inhibitor elaborated by C. michiganense was different from that elaborated by the antagonistic strain of C. insidiosum for the reason (1) that the inhibitor elaborated by two of the cultures of C. michiganense was markedly active on both the antagonistic and non-antagonistic strains of C. insidiosum while the inhibitor from the antagonistic strain of C. insidiosum was not; and (2) that the inhibitor elaborated by the other two cultures of C. michiganense was markedly active against one culture of the non-antagonistic strain of C. insidiosum and faintly active against the other culture of the non-antagonistic strain and the one culture of the antagonistic strain of that species.

To confirm or refute this apparent difference in the inhibitors from C. michiganense and C. insidiosum, the filtrates of the four C. michiganense cultures were tested for inhibitory properties against culture L of the non-antagonistic strain of C. insidiosum which had responded differently to the four cultures of C. michiganense. At the

TABLE XXXV. INHIBITION OF STRAINS OF *C. INSIDIOSUM* BY STRAINS OF *C. MICHIGANENSE*

Spot-planted Strains	Zone Width (mm.) in Agar[a] Seedings of:						
	C. michiganense strains				*C. insidiosum* strains		
	9	4450	7429	102	145(A[b])	96(N[c])	L(N)
C. michiganense 9	0	1	0	0	8	10	10
C. michiganense 7429	0	0	0	0	8	14	10
C. michiganense 4450	1	0	0	0	2	6	1
C. michiganense 102	0	0	0	0	1	10	1

[a]Burkholder's agar.
[b]Antagonistic strain.
[c]Non-antagonistic strain.

same time portions of the filtrates of the four cultures of C. michiganense were heated to see whether or not this treatment of them would reveal similarities or dissimilarities to the inhibitor of C. insidiosum. As seen from the results for this test in Table XXXVI, conducted on peptone-deficient Burkholder's agar medium, filtrates from all of the C. michiganense cultures inhibited the non-antagonistic strain of C. insidiosum with heated filtrates from three cultures of the former species being less or no more effective than the unheated ones. The heated filtrate from the other culture of C. michiganense was almost three times as effective as the unheated filtrate as was also true of the heated filtrate of the antagonistic strain of C. insidiosum. Therefore, from the results of this test coupled to those from the preceding one, one may conclude that the inhibitors elaborated by the four cultures of C. michiganense were different from the one elaborated by the antagonistic strain of C. insidiosum, even though the inhibitor from one culture of C. michiganense responded to heat treatment in the same manner as the inhibitor from the antagonistic strain of C. insidiosum.

As a final test of the similarities and dissimilarities between the two groups of inhibitors, heated and unheated filtrates from single cultures of the antagonistic and non-antagonistic strains of C. insidiosum were tested against peptone-deficient Burkholder agar seedings of the four cultures of C. michiganense and of the same C. insidiosum cultures supplying the filtrates. As may be seen from the inhibitory zone widths recorded in Table XXXVII, the filtrate from the antagonistic strain of C. insidiosum noticeably inhibited only the non-antagonistic

TABLE XXXVI. INHIBITION OF C. INSIDIOSUM L BY AGAR CULTURE FILTRATES OF C. MICHIGANENSE AND C. INSIDIOSUM 145

Agar Culture Filtrate of:	Zone Diameter Produced by Filtrates	
	Unheated	Heated[a]
C. michiganense 9	18	18
C. michiganense 4450	13	8
C. michiganense 7429	14	9 (faint)
C. michiganense 102	9	25
C. insidiosum 145 (A[b])	8	26

[a]Heated at 100°C for 1 minute.
[b]Antagonistic strain.

TABLE XXXVII. INHIBITION OF STRAINS OF C. INSIDIOSUM AND C. MICHIGANENSE BY AGAR-CULTURE FILTRATES OF C. INSIDIOSUM

Agar Seeded With:	Zone Diameter (mm.)			
	C. insidiosum L Filtrate		C. Insidiosum 145 Filtrate	
	Unheated	Heated[a]	Unheated	Heated
C. insidiosum L (N^a)	0	0	8	25
C. insidiosum 145 (A^b)	0	0	0	0
C. michiganense 9	10	8	sl. inhib.	sl. inhib.
C. michiganense 7429	sl. inhib.	sl. inhib.	sl. inhib.	sl. inhib.
C. michiganense 4450	0	0	0	0
C. michiganense 102	0	sl. inhib.	0	0

[a]Heated at 100°C for 1 minute.
[b]Non-antagonistic strain.
[c]Antagonistic strain.

strain of the same species, did not inhibit two of the cultures of C. michiganense and only slightly inhibited the other two cultures of C. michiganense. The two cultures of C. michiganense that were slightly inhibited in this test were the same that markedly inhibited the culture of the antagonistic strain of C. insidiosum in a previous test (Table XXXV), while the other two cultures of C. michiganense that were not inhibited in this test were the same that slightly inhibited the antagonistic strain of C. insidiosum in that same previous test. Hence from such comparisons one may submit these differences as further evidence for the dissimilarities between the inhibitors produced by cultures of C. michiganense and the antagonistic strain of C. insidiosum. Furthermore, additional evidence for the dissimilarity of the inhibitors from the two species may be derived from Table XXXVII from the fact that the heated filtrate of the antagonistic strain of C. insidiosum was more inhibitory than the unheated filtrate only to the non-antagonistic strain of the same species and not to cultures of C. michiganense.

In addition to the different inhibitors produced by cultures of C. michiganense and by the antagonistic strain of C. insidiosum, one may also note from the data in Table XXXVII that the non-antagonistic strain of C. insidiosum also produced an inhibitor that was active against culture 9 of C. michiganense and only slightly active against culture 7429 of the latter species. Contrariwise, in a previous test (Table XXXV) the same cultures of C. michiganense markedly inhibited the non-antagonistic strain of C. insidiosum. Moreover, the inhibitor from the non-antagonistic strain of C. insidiosum failed to respond to heating in the

same manner as the inhibitor from the one strain of C. michiganense (culture 102) and the one from the antagonistic strain of C. insidiosum. Therefore, the inhibitor elaborated by the non-antagonistic strain of C. insidiosum may be considered to belong to another group of inhibitors.

In summary, the inhibitor from the antagonistic strain of C. insidiosum had a narrow spectrum in that it affected only the non-antagonistic strain of the same species, the serologically related species of C. michiganense, and only slightly inhibited a culture of C. sepedonicum. The close relationship between C. insidiosum and C. michiganense was emphasized by the rare phenomenon of thermal activation of the inhibitor not only in the antagonistic strain of C. insidiosum but in a culture of C. michiganense as well, even though the inhibitors elaborated by these two strains were proven to be different.

DISCUSSION

Persistence of *Corynebacterium insidiosum* in Soil

The rapid vitiation of *C. insidiosum* in moderately moist soil at above-freezing temperatures, noted in the present study, raises doubt on one of two items; namely, (1) that the soil is a reservoir of wilt bacteria for the local and widespread incidence of bacterial wilt of alfalfa, and (2) that the experimental conditions were fully representative of those in the field. There is no certain knowledge, at present, as to the source of bacteria that start the disease nor as to how the disease starts in the field; that is, whether the disease starts at one or several "select" loci over the field, or if it starts at many loci uniformly over the field. If wilt bacteria were brought into a field with planted seed (4), then one might expect many infection loci over the field. However, if wilt bacteria were derived from the soil, then one might expect them to be confined to certain spots or areas in the field that had favored persistence of the bacterium. To recognize such contrasting situations one would have to examine fields before the mower bar had a chance to spread the bacteria from plant to plant and, thereby, obliterate the contrast. The fact that the disease often makes its first appearance in a severeform in certain portions of a field, rather than over an entire field simultaneously, lends some support to the idea that the bacterium is soil borne. However, such localizations of severe disease may be expressions of more favorable conditions for disease development in the plant in those areas than in other parts of the field.

The fact that the disease occurs in nearly every alfalfa field in the humid and semi-humid areas of the country lends support to either or both of two ideas, namely, that the bacterium is introduced with the seed into each of the fields, and that the bacterium survives in the field soil from one alfalfa crop to the next, regardless of the time between alfalfa crops. As nothing is known of the extent of seed or seedling infection by seed-borne wilt bacteria, the evidence at the moment is that the soil is the primary source of the bacterium and that the bacterium persists there indefinitely, probably in certain favored areas. In view of this, therefore, the second item mentioned above is open to doubt, namely, that the experimental conditions of the present study were fully representative of those in the field. In this study the soil microflora was shown to exert an important influence on the ability of the wilt bacterium to persist in the soil and, therefore, any factor that influences the activity of this microflora would be expected to influence the ability of the wilt bacterium to persist in the soil. The alfalfa wilt bacterium persisted in relatively undiminished numbers for longer than 5.5 months in soil near the wilting point and at temperatures below freezing, and in frozen moist soils for the same length of time. It persisted longer in some soil samples than in others, probably because of a difference in composition of the soil microflora. In moist soil at above-freezing temperatures, on the other hand, the wilt bacterium persisted for only a few days, which essentially is what Lee (38), Fulton (15), Peltier and Frederich (47) and Loucks (39) found in studies of Xanthomonas citri (Hasse) Dowson, and Grogan and Kendrick

(20) reported for C. michiganense. The centers or spots within a field where C. insidiosum may persist, therefore, must be those where the soil microflora is relatively inactive by virtue of the numbers or kinds of microorganisms present. Such centers or spots may be the rhizospheres of plant roots, the region below the plow line or surface-dry soil.

An Antagonistic Strain of Corynebacterium insidiosum

The antagonistic strain of C. insidiosum found in the present study may now be added to the list of strains already reported for that species. Rosenthal and Cox (54) reported two serologically different strains, Fulkerson (13) found a white pigmented form in association with the usual blue pigmented ones, Fulkerson (12, 14) reported isolates with a differential virulence to clones of alfalfa and Cook and Katznelson (3) revealed three strains differing in susceptibility to bacteriophages.

When the antagonistic strain of C. insidiosum was first observed, the writer considered the inhibitory principle to be a conventional antibiotic. However, as many antibiotics are readily produced on minimal media and affect a wide range of microorganisms, the suggestion was made that the inhibitor more closely resembled the colicins elaborated by the coliform group of bacteria than the conventional antibiotics.[1]

The properties of some colicins produced by E. coli as revealed by Gratia (18), Gratia and Frédéricq (19), Jacob et al (27), Frédéricq

[1]The author is indebted to Dr. H. E. Calkins, Bacteriology Department, South Dakota State College, for this suggestion.

(11) and Heatley and Florey (23), are listed in Table XXXVIII along with properties of the inhibitor elaborated by the antagonistic strain of C. insidiosum. Generally, the properties of the inhibitor of the antagonistic wilt bacterium were similar to the properties of the colicins. Most of the colicins investigated were thermostabile as was the inhibitor of the antagonistic strain of C. insidiosum; however, the inhibitor of the latter was capable of being thermally activated while those of the former were not. Aeration did not inactivate the inhibitor in the unheated filtrate from an agar culture of the antagonistic strain of C. insidiosum. When the same filtrate was heated and then aerated, the inhibitor was completely inactivated. Probably some change occurred in the configuration of the inhibitory principle of the antagonistic wilt bacterium after heating; nevertheless, the inhibitor from both the heated and unheated filtrates was still precipitated by acetone which might indicate that the change in configuration upon heating was not so great as to change the inhibitor's specificity of action toward the non-antagonistic strain of C. insidiosum and toward cultures of C. michiganense.

Compounds similar to the colicins are produced by certain strains of Pseudomonas pyocyanea (≡ Pseudomonas aeruginosa (Schroeter) Migula), and their range of action seems restricted to the family Pseudomonadaceae according to Jacob (25). Furthermore, another colicin-like compound is produced by Bacillus megaterium deBary and was called "megacine" by Ivánovics and Alföldi (24). The general term "bacteriocins," which would include the colicins, was proposed by Jacob et al.(26) to include

TABLE XXXVIII. PROPERTIES OF THE INHIBITOR PRODUCED BY THE ANTAGONISTIC STRAIN OF C. INSIDIOSUM AS COMPARED TO COLICINS PRODUCED BY E. COLI

Property of Inhibitor	Colicins	Inhibitor of Antagonistic Strain of C. insidiosum
Reproduction of inhibitor	-	-
Produced by cells sensitive to antagonistic strains	-	-
Water soluble	+	+
Diffusible in agar	+	+
Precipitated by acetone	+	+
Resistant to action of chloroform	+	+
Sedimented by centifugation at 17,000- 20,000 G	- (20,000 G)	- (17,000 G)
Thermostabile	+	+
Thermally activated	-	+
Produced in liquid media	-	-
Passage through Seitz filter	-	-
Complex media required for production	+	+
Action restricted to members of same or related species	+	+

\+ positive for any given property.
\- negative for any given property.

inhibitors of a protein nature whose effect is lethal and whose range of action is restricted to related strains or species. While the inhibitor elaborated by the antagonistic strain of C. insidiosum was bacteriostatic rather than bactericidal, many of its properties are similar to the colicin-like compounds and tentatively it may be classed with the bacteriocins.

The other inhibitors encountered in the four different cultures of C. michiganense and the one non-antagonistic culture of C. insidiosum may be other bacteriocins. To demonstrate their existence, however, a wide variety of strains would have to be collected and be tested against each other in all possible pairs.

The antagonistic strain of C. insidiosum might prove to be a useful tool in genetical studies of the C. insidiosum and C. michiganense group, and might also serve a useful purpose in taxonomical studies of the corynebacteria.

SUMMARY

Studies were conducted on the persistence in soil of Corynebacterium insidiosum, the alfalfa wilt bacterium, and on an antagonistic variant of that species. The persistence was followed by plate counting colonies from soils heavily inoculated with cells of this bacterium. The antagonistic variant of the species was studied directly and from cells or culture filtrates spot-planted on agar media containing seedings of other bacteria.

Most cells of the wilt bacterium remained viable on glass surfaces for over 7 months at different temperatures, and in moist sterilized soil for over 72 days at room temperature.

The cells persisted for only a few days in moist non-sterilized soil at room temperature, for 66 days in moist sterilized and non-sterilized soil at freezing or slightly-above freezing temperatures, and for over 5.5 months in very dry or moist soil at below-freezing temperatures.

The bacterium persisted for a longer period in some soils than in others, regardless of the texture or pH of those soils. It persisted longer in moist sterilized soil than in a similar soil contaminated with a small amount of non-sterilized soil or a streptomycete. Soil microorganisms thus were considered responsible for the rapid decline of C. insidiosum in soil, with streptomycetes probably contributing to the decline.

The wilt bacterium did not persist over 30 days in wilt-infected alfalfa roots stored in a moist non-sterilized soil at room temperature.

The antagonistic variant of C. insidiosum was proven such from morphological, cultural, serological, pathogenic and antibiotic sensitivity tests. It inhibited other cultures of C. insidiosum, but not itself. The other cultures of C. insidiosum were non-inhibitory to the antagonistic variant or to themselves; hence, such cultures were termed non-antagonists.

The inhibitory property of the antagonistic variant was exhibited by all cells of a culture, and it was stable on passage of the bacterium through alfalfa roots alone or in combination with a non-antagonistic culture of C. insidiosum. The antagonistic and non-antagonistic cells were mutually compatible in such roots.

The inhibitory intensity of the antagonistic variant varied among cells re-isolated from alfalfa roots where non-antagonistic cells were present. The intensity was less from old than from young cultures, less on peptone-containing than on peptone-deficient Burkholder's agar medium, less on heavily seeded than on lightly seeded Burkholder's agar medium, less on normal than on reduced agar concentrations in Burkholder's medium, and less at well-below optimum than at optimum incubation temperatures.

The inhibitory principle was extracellular, water-soluble and diffusible through agar. It was contained in a filtrate from Burkholder's agar medium but not from Burkholder's broth medium. It passed through a fritted-glass but not a Seitz filter. The principle was precipitated by acetone, resistant to chloroform, not sedimented by high-speed centrifugation, and thermostabile over a pH range of 3.0 to 8.2. Short stemmings of the filtrate doubled or tripled the effectiveness of the inhibitory

filtrate, and rendered it susceptible to inactivation by aeration.

The inhibitory range of the antagonistic variant was limited to cultures of C. insidiosum, C. michiganense and C. sepedonicum. One non-antagonistic culture of C. insidiosum slightly inhibited cultures of C. michiganense. Four cultures of C. michiganense markedly or slightly inhibited the antagonistic variant and non-antagonistic cultures of C. insidiosum. The inhibitory effectiveness of a filtrate of one culture of C. michiganense was more than doubled by a short period of steaming.

The inhibitor of the antagonistic strain of C. insidiosum was tentatively classed with the bacteriocins because of its similarity to this group of compounds.

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